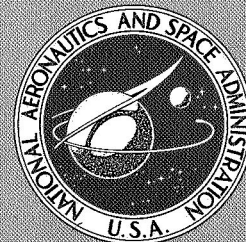


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# **CASE FILE** **BIOLOGY OF HUMAN TISSUE**

Proceedings of the  
Second Conference

PACIFIC PALISADES, CALIFORNIA  
MARCH 6-9, 1966



NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

# BIOLOGY OF HARD TISSUE

## Proceedings of the Second Conference

Edited by  
ANN M. BUDY

This conference was sponsored by the National Aeronautics and Space Administration, the Office of Naval Research, and The New York Academy of Sciences. It was held at Pacific Palisades, California, from March 6 to 9, 1966.



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## PREFACE

The Conference which this publication attempts faithfully to report had as its purpose a discussion in depth of topics that are increasingly challenging the attention of scientists in many fields. Perhaps, then, it is not inappropriate that I present at the outset a few of my basic assumptions. The major one is that nature is all one piece. The second one is that as we get more information, more data flowing in at an increasingly rapid rate, we are faced with the realization that the area of ignorance is much larger than we thought. Wouldn't you agree with me that the half-life of facts is getting shorter and shorter?

There is almost no systematic effort made to provide for interdisciplinary communication, nor multidiscipline discussion. If what I am saying is true about the rate of inflow of new information, if we have reached the point—and I think we have—where no one ever again can claim that he is up-to-date even in the narrowest area of science, then we must accept the fact that never again can anyone fool himself into feeling that he knows the last word in any science, no matter how narrow. What we have to do is accept a limitation in having only a relative sample of what is going on in any particular area; and if we wish to participate in the breakthroughs or to understand them when other people make them, we must also have a relative sample of the data from disciplines related to our area of special concern. At the present time there is little systematic effort to make this possible. This is what we are about, and we think it terribly important, because we believe that this is the essence of science.

We believe that the standard publications really falsify science, because they give a logical progression but this was not the way the research was done. The research went here and there, and started over here again with another idea, and finally it ended up here; but the man who started this research left a little flag where he began and now he draws the line back to that little flag and this is what he publishes—a simple, straight, logical process.

This is not the way it happened. It is to some degree a false picture of science. It fools us and it fools our students and discourages really imaginative students from attempting anything as dull as a purely logical process. Logic is very important, but it is not the creative aspect.

We used to be brought up with the idea that one observes phenomena and then takes a second step and interprets it, but I think there is a great deal of evidence from many sides to show that this is not the case, really—that what we do is to build an interpretation directly into

the initial observation. Therefore, all observations are really interpretations based on observations in our own past. It is impossible for us to be a tabula rasa; we always see anything we perceive in the light of something in our past, so we are trailing clouds, not of glory, but of the past as we approach each new phenomenon. This means that all of us put our special coloring of our own past experience on what we see or hear or observe, and, therefore, all of us put, to some degree, a false-ness, a distortion upon our observations. It is very important that we become aware of this and, instead of denying it, take account of it and do such things as can be done to counterbalance it.

These distorting lenses and deaf spots and blind spots that we carry around with us, however, are not absolutely fixed objects. You can see the distorting lenses bending in the presence of hostility or anxiety, or you can see the lenses flattening out—people may even take them off and put them down on the table—if you can set up the appropriate atmosphere (and we hope we have). The blind spots will disappear, the deaf spots will shrink, and you will see each other and suddenly begin to understand each other.

FRANK FREMONT-SMITH, DIRECTOR  
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## INTRODUCTION

*The Second Conference on Biology of Hard Tissue, under the auspices of the New York Academy of Sciences Interdisciplinary Communications Program, was held at Pacific Palisades, Calif., with Dr. Marshall R. Urist and Franklin C. McLean as Cochairmen.*

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URIST: It is a great pleasure to welcome you to Los Angeles and suburban Pacific Palisades. Science is constantly moving and changing; hopefully, this conference will change present knowledge of bone and stimulate that knowledge to move in the right direction.

I am going to ask Dr. Franklin McLean to open this conference.

MCLEAN: I have nothing further to add to the statement that Dr. Urist has made. I expect to have a good deal to say before this conference ends, but I would prefer to hold my remarks until later.

URIST: Thank you very much, Dr. McLean. I will ask Dr. Frank Fremont-Smith to read some remarks that he made in 1953 on the occasion of the last Macy Foundation Conference entitled "Metabolic Interrelations," which dealt with calcium and bone metabolism. The First Conference on Biology of Hard Tissue at Princeton last year and this conference are a continuation of the Macy conference program. Dr. Fremont-Smith is the person who organized the Macy Foundation Interdisciplinary Conferences, and I would like him to read his remarks.

FREMONT-SMITH: Thank you. Upon the orders of my Chairman, I will read the following.

As an introduction to these Transactions of the Fifth Conference on Metabolic Interrelations, I should like to outline what it is that the Foundation hopes to accomplish by its conference program. We are interested, first of all, in furthering knowledge about metabolic interrelations, and to this end the participants were brought together to exchange ideas, experiences, data, and methods. In addition to this particular goal, however, there is a further, and perhaps more fundamental aim which is shared by all our conference groups. This is the promotion of meaningful communication between scientific disciplines.

The problem of communication between disciplines we feel to be a very real and urgent one, the most effective advancement of the whole of science being to a large extent dependent upon it. Because of the accelerating rate at which new knowledge is accumulating, and because discoveries in one field so often result from information gained in quite another, channels must be established for the most effective dissemination and exchange of this knowledge.

The increasing realization that nature itself recognizes no boundaries makes it evident that continued isolation of the several branches of science is a serious obstacle to scientific progress. Particularly is it true in medicine that the limited view through the lens of one discipline is no longer enough. For example, today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation. At the other extreme, medicine is certainly a social science, and, through mental health, must be concerned with economic and social questions. The answer, then, is not further fragmentation into increasingly isolated specialties, disciplines, and departments, but the integration of science and scientific knowledge for the enrichment of all branches. This integration, we feel, can be encouraged by providing opportunities for a multiprofessional approach to given topics.

Although the fertility of the multiprofessional approach is recognized, adequate provision is not made for it by our universities, scientific societies, or journals. And perhaps the presence of other hindering factors must be admitted. Partly semantic in nature, they may also to some degree be psychological. Admittedly, it is oftentimes difficult to accept data derived from methods with which one is unfamiliar. By making free and informal discussion the central core of our meetings, we hope to achieve an atmosphere which minimizes as much as possible these semantic and emotional barriers.

Thus, our conferences are in contrast to the usual scientific gatherings. Presentations are not designed to present neat solutions to tidy problems, but rather to elicit provocative discussion of the difficulties which are being encountered in research and practice. We ask that the presentations be relatively brief and emphasis is placed upon discussion as the heart of the meeting. Our hope is that the participants will come prepared not to defend a single point of view but, with open minds, to take full advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way as they talk with their own colleagues in their own laboratories.

When a new conference group is organized, the Chairman, in consultation with the Foundation, selects 15 scientists to be a nucleus of the group which will hold annual meetings for a period of 5 years. Every effort is made to include representatives from all the pertinent disciplines. From time to time, however, new members are added by the group to fill gaps in viewpoint or technique. A small number of guests is invited to attend each meeting, but, for the purpose of promoting full participation by all members and guests, attendance at any meeting is limited to 25.

The Transactions of these meetings are recorded and published. This is done because the Foundation wishes to make current thinking in a field available to all those working in it.

Logic is a vital aspect of science, but equally essential is the intuitive or creative aspect. Research is as creative as painting a portrait or composing a symphony. Although logic is, of course, necessary in order to rearrange, to test, and to validate, research thrives on creativity which has its source in unconscious, nonrational processes. Unfortunately, however, in the research reports which are presented to the world in scientific journals, this integral part of scientific endeavor is shriveled by the cold, white light of logic. By preserving the informality of our conferences in the published transactions, we hope to portray more accurately how the minds of scientists work and to give a true picture of the role which creativity plays in scientific research [ref. 1].

The conferences are now being conducted under the auspices of the New York Academy of Sciences, and I am very happy to welcome you on behalf of the New York Academy of Sciences to this series, which is a continuation of the basic principles of the Macy Foundation Conferences.



URIST: Thank you very much, Dr. Fremont-Smith. It is the custom of the conference program for each conferee to introduce himself. Now, Dr. Copp, will you begin with the introductions?

COPP: I suspect that I have been asked to begin the introductions because I am one of the oldest of the young rebels in the bone field. Although some of my remarks may be a repetition of those made at Princeton in 1965, some of you may be interested in how I originally became interested in bone physiology. In 1941 I was working with Dr. David Greenberg at the University of California on the uptake of radioactive calcium by healing bone fractures. About that time, interest developed in the possible toxicity of two new bone-seeking isotopes—plutonium and  $^{90}\text{Sr}$ . I was assigned the problem of removing these elements from bone without harming the animal (ref. 2). I found the problem insoluble, but the skeleton fascinating. We did find that radiostrontium could be removed from the bones of young rats by feeding a diet deficient in phosphorus (ref. 3), but in this process almost all the mineral was removed from the skeleton. Indeed, the bones became rubbery, the vertebral column and rib cage collapsed, and the animal died a respiratory death because of structural skeletal failure. We are still interested in the effects of dietary calcium and phosphate on bone which I shall discuss later in the meeting.

I presented our work on severe phosphorus deficiency at a meeting in Chicago of the Federation of American Societies for Experimental Biology (ref. 4). Two members of that audience were to have a profound effect on my scientific future: Dr. A. Baird Hastings and Dr. Franklin C. McLean, who spoke to me after the meeting and encouraged me to continue our work on bone. This inspiration was reinforced by an invitation to attend the 1951 Macy Conference on Metabolic Interrelations. At the final Macy Conference in 1953, a very inspiring talk by John Eager Howard on calcium homeostasis (ref. 5) stimulated me to present a paper on this subject at the first Gordon Research Conference on Chemistry, Physiology, and Structure of Bones and Teeth in 1954. It is evident from the program of this meeting that I am still interested. I must confess that the problem seems much more complicated than it appeared in 1954 when there was only one hormone to worry about; however, I feel that we are following the path set for us by Dr. McLean and Dr. Hastings many years ago.

WALSER: Very briefly, I am in the Departments of Medicine and Pharmacology, Johns Hopkins University, and am very pleased to be here again. I think this approach to increasing understanding is an excellent one. I am interested in ionic interactions, interactions between ions in solution, particularly ion pairs and, more recently,

interactions between like-charged ions in metabolism at the subcellular level, the cellular level, and finally at the level of the whole organism.

HOWELL: After several years at the National Heart Institute, Bethesda, Md., studying the electrolyte metabolism in dogs, I became interested in the partition of electrolytes in cartilage and bone, particularly mechanisms of calcification as approached by ultramicrobiochemical methodology which we have developed in my laboratory together with Dr. J. Pita and Dr. J. Marquez. Also, I have spent 2 years at the Karolinska Institutet, Stockholm, working on the application of microscopic X-ray elemental analysis for sulfur and phosphorus to the study of endochondral ossification.

BAUER: I am an orthopedic surgeon particularly interested in the etiology of fractures, especially in old people. As a clinician, I am interested in progress in the basic sciences and try to understand what is in it for us. I hope it will be possible one day to prevent fractures in the aged. I feel this is particularly important because it is unlikely that therapy in this field will ever efficiently solve the very severe sociologic problem posed by certain fractures in the aged, notably those of the upper part of the femur. I have studied epidemiology of fractures to understand more about age changes in bone and am interested in tracer studies of bone mineral metabolism because they present quantitative data for protein synthesis and breakdown.

PRITCHARD: At present I am in the Department of Anatomy, University of Illinois, at the Medical Center in Chicago, on a year's leave of absence from Queen's University, Belfast, Ireland. I want to know where osteoblasts come from.

My interest in bone started when I was looking for a research problem in 1939; Hugo Gray, my professor, suggested that I try to find out why the sutures in the skull are where they are. It took me a long time to find out, and I got involved in a lot of other bone problems on the way. I have been particularly concerned with growth and repair mechanisms in the skeleton. I have recently become a chicken doctor, since chickens get bigger and grow faster than rats.

OWEN: I entered this field, like Dr. Copp, at an early stage of the interest in  $^{90}\text{Sr}$  and bone. I joined the Medical Research Council, Bone-Seeking Isotopes Research Unit, Churchill Hospital, Oxford, under Dr. Janet Vaughan and was directed to work on the effects of radiostrontium on bone. As a physicist, naively, I thought that everyone knew how bone worked and that it was just a question of investigating the effects of  $^{90}\text{Sr}$  on it. I spent several years studying  $^{90}\text{Sr}$  dosimetry in bone and the relationship between radiation dose and damage following different patterns of administering  $^{90}\text{Sr}$  to rabbits. During this time my interest in the biologic side increased, and I decided to study bone cells. I studied cell population kinetics of growing

osteogenic tissue using labeled thymidine and glycine in conjunction with autoradiographic techniques. Recently, I have been interested in the pattern of RNA metabolism in the different functional states of bone cells. My main interests are cell differentiation and hormone effects as applied to bone. I have spent 2 years in the United States, one in the Donner Laboratory of Biophysics, Berkeley, Calif., and one at Brookhaven National Laboratory, Upton, L.I., N.Y.

HEANEY: I am an internist interested in those factors that control the balance between bone formation and bone resorption in health and disease and how these are related to the production of metabolic bone diseases. I am interested in calcium kinetic techniques that can be used as tools to get at these problems.

CURREY: I seem to be the first one, perhaps the only one, who is not wholly wedded to bone. All my work is on hard tissue because about a third of my time is spent on snail shells. It is not the quality of the shell that interests me, but simply the genetics it displays. I am interested in the evolution of certain characteristics in snail populations. Apart from that, I have a somewhat zoologic interest in hard tissues, all sorts of hard tissues apart from bone, namely echinoderm skeleton, mollusk skeletons, and so on. My main interest is their mechanical properties, their histologic picture, their structure as we know it from all sorts of studies, how it fits in with the mechanical properties, and how the particular mechanical properties that particular kinds of skeletons have are of adaptive significance for the animals.

SAXÉN: I am representing the University of Helsinki and am, perhaps, the only one who is mainly interested in soft tissues. Since 1952 my group has been working on a problem called tissue interaction, which refers to the mechanism that controls tissue differentiation.

To study this problem one must build certain model systems; we have been particularly interested in two of them. One is the development of the central nervous system; the other, a more recent one, is the development of mammalian kidney. In both these models we are dealing with inductive tissue interaction. I hope that this problem is related to what is now known as bone induction.

Only recently have we worked on some hard tissues as well. We have been interested in organotypic tissue culture, and we are cultivating embryonic bones in order to analyze the possibilities of testing drug action on bones in these organotypic cultures.

At present we are working with tetracycline. It is still a sideline to the basic studies on the tissue interaction, but perhaps after this meeting I will know more about it.

BÉLANGER: I am from one of the smaller medical schools in Canada and have had a variety of interests. Immediately after the war, during which I served as a clinician in the Royal Canadian Air Force, I

returned to McGill with my friend, Charles Leblond; at that time the radioisotopes, or at least a few of them, were here. One radioisotope was phosphate, or phosphorus; another was  $^{131}\text{I}$ . Experimenting with these two, which we could obtain only in sparse amounts, we tried to make dynamic histologic studies with the methods of autoradiography (coating technique).

Leblond decided to follow through with iodine; I followed through with phosphate and then calcium, using this newly acquired tool and working first on problems of growth and accretion and then on problems of resorption, in which I am interested at the moment. Along the line such tools as methods of histochemistry, specialized methods of microradiography, and electron microscopy allowed us to see things a little clearer than with standard methods of histology. I am greatly indebted all through this time, over 20 years, to the people with whom I have been associated: Charles Leblond, Harold Copp, and many others.

HOLTZER: My main interest is cell differentiation. I see this as a problem of decision making—how a cell elects to engage in one series of processes to the exclusion of other processes. Although all cells make cytochromes, cells producing albumin do not normally synthesize myosin. A corollary to this is the question: when embryonic cells divide, how is it they do not perpetuate their parental phenotype? As such, my questions are not exclusively aimed at hard tissues, although I have worked with cartilage cells.

SJÖSTRAND: I am from UCLA and am interested in the functional significance of the structural organization of cells, particularly at the molecular level. I have particular interest, with respect to calcification, in the structural factors involved in nucleation, crystallite formations, and so on.

RAISZ: I am from the University of Rochester. I became interested in kidney disease and kidney physiology while working with Homer Smith and then drifted into the field of calcium metabolism, largely because of clinical dissatisfactions similar to those expressed by the Macy Foundation group in 1953. This dissatisfaction with our knowledge of clinical bone disease ultimately led me to work in tissue and in organ culture with embryonic bone and parathyroids, and to studies of parathyroid hormone action. My particular interest is in the parathyroid feedback mechanism.

WHEDON: Since 1952 I have been at the National Institute of Arthritis and Metabolic Diseases in Bethesda, Md., carrying on my particular interest in mineral metabolism with various techniques, notably metabolic balance and radioisotopic kinetic studies. Despite some added administrative chores since 1962, I still keep active in

the Mineral Metabolism Section of that Institute, now headed by Gerald Aurbach.

During the past year, I have had the very interesting experience of organizing and attempting to carry out the first metabolic balance study in space. With the considerable assistance of Leo Lutwak, who was with me at the National Institutes of Health and is now at Cornell in Ithaca, New York, and some very necessary help from the NASA staff, we were able to carry out a study on the Gemini VII flight in December. We achieved a limited, but valid control phase before the flight, a control of intake collections during flight, and a very brief postcontrol phase thereafter. The data are beginning to come out, and possibly within the next few months we will be able to report on this very difficult project.

PECK: I have been in research only 5 years. I started out with an interest in connective tissue metabolism, working under Dr. William H. Daughaday, Washington University School of Medicine, St. Louis. I was fortunate then to spend 2 years under the kind and astute aegis of Dr. Whedon. For the past 8 months, I have been at the University of Rochester in the Department of Internal Medicine. My primary interest is in the biochemical differentiation of bone cells.

ROBINSON: I am an orthopedic surgeon. It is interesting to hear that 5 of the 25 participants here are or have been associated with the University of Rochester; I think that this is in some way a tribute to Drs. Harold Hodge and William Neuman, who have been so active in dealing with problems of bones and teeth.

My interest began when I wanted to discover the structure of bone (refs. 6 to 10). As an orthopedic surgeon I had to work with this material and thought that we ought to know more about this structure. Later we became interested in the bone cell because this cell produces the mineralizable bone matrix, which has a very even quality in normal members of various families of animals. Now we have come to the conclusion that these cells are under controls beyond themselves.

LLOYD: As a pure physicist, like three of the British representatives here, I was associated with the University of Belfast, Queen's University. Later I went to London and worked on the standardization of radioisotopes at the National Physical Laboratory. While I was there, I joined the Radiology group and became interested in tracer techniques; this work led me into St. Bartholomew's Hospital, London, where I studied all the tracers used clinically in a hospital. I later worked with Dr. Janet Vaughan's group, the Bone-Seeking Isotope Research Unit, in Oxford. At that time the main interest of this group was a study of  $^{90}\text{Sr}$  from fallout, particularly in bone. While working with rabbits, I observed that strontium metabolism seemed closely



associated with calcium metabolism and instigated a research project to study the comparative metabolism of strontium and calcium in rabbits. I also became interested in the human radium cases and am grateful to Dr. Hasterlik for providing small portions of bone on which I made autoradiographic and microradiographic studies. Last year, I spent 8 months at the Argonne National Laboratory with Bob Rowland and John Marshall. I worked with John Marshall on the development of an automatic computer method for the study of autoradiographs and microradiographs of bone. Now I have returned to Oxford and am back working on the differences in strontium and calcium metabolism, with particular emphasis on the reasons for the discrimination between blood and bone.

ROWLAND: I am a biophysicist from the Argonne National Laboratory. My present interests in bone have to do with the uptake and retention of isotopes in bone, and, in particular, bone turnover processes.

I entered this field as a consequence of my studies of radium in the human being. Where is radium in bone? How long does it stay there? To answer these questions, the techniques of autoradiography and microradiography were applied to radium bones. In the midfifties Dr. McLean tried to teach a few physicists what was happening in this fascinating tissue, and my experience in this group represents my real contact and education in bone. In the sixties, I was fortunate to spend 2 years at Rochester with Bill Neuman and his group learning about the Rochester approach to bone. Since that time I have again returned to Argonne, and my present interests are leading me quite specifically toward the role of the osteocyte in bone. What does the osteocyte do? What is the role of this most intriguing cell and its canalicular pathways? I am hoping to use isotope techniques to learn more about the processes that occur in this most unusual cell.

ARNAUD: I am a physician-endocrinologist with 3 years postdoctoral training in biochemistry. For the past 4 years, I have been with Dr. Howard Rasmussen, first in the Department of Biochemistry at the University of Wisconsin and now in the School of Medicine at the University of Pennsylvania. Our research has been related primarily to the chemistry and mechanism of action of the parathyroid hormone. However, thanks to Dr. Copp, we have become interested in a second hormone, thyrocalcitonin, which has a profound influence on calcium and phosphate homeostasis. The isolation and chemistry of this interesting polypeptide has occupied most of our time, but recently I have developed a radioimmunoassay for its measurement in biologic fluids. We have employed the purified material in physiologic studies of its mode of action and relationship to parathyroid hormone.

BUDY: I am Dr. McLean's research associate and have been associated with him for the past 20 years. By training I am a pharmacolo-

gist, but I have been working in the general field of physiology of bone. Thus far it seems that this group has expressed specialized interest in the osteocyte and the osteoblast. But is there no longer any specialized interest in the osteoclast?

URIST: Dr. McLean, what brought you into the field of bone?

MCLEAN: I have been in this field a long time. I was brought into it first by A. Baird Hastings in connection with the ionization of calcium in protein-containing solutions, particularly serum. I have studied the entire field of the physiology of bone and calcium metabolism. If I were asked to pinpoint any one particular thing that I have paid more attention to than anything else over the years, it would be the relation of the parathyroid hormone to bone. But that by no means limits my interests.

URIST: Dr. Fremont-Smith, why did you start all your activity in the field of bone?

FREMONT-SMITH: I was trained in neurology, neuropathology, and in body fluids. I started out with cerebrospinal fluid when I was a medical student and worked with it for a good many years. However, I became more and more interested in the influence of emotional factors on physiologic processes, and moved into psychosomatic and psychiatric interests. I am now with the New York Academy of Sciences, still running the same kind of conference that I used to when I was in the Macy Foundation.

URIST: We are very grateful for your continuing interest in this field, Dr. Fremont-Smith.

Dr. Talmage, what brought you into the field of calcium and bone?

TALMAGE: In graduate school, I was trained as a reproductive physiologist, and then I accepted a position at Rice University. I started in this field, however, when I had an Atomic Energy Commission contract to work with radioisotopes soon after the war when this field was rapidly expanding. One of the first isotopes I used was  $^{45}\text{Ca}$ , and this stimulated my interest in calcium. I decided to try to prove that the Albright and Reifenshtein theory of parathyroid action was wrong. For 10 years I studied this parathyroid action as it was related to kidney physiology. Any study of parathyroid function naturally leads to bone, since this is the site of its primary action. It did not take me long to realize that the bone field was an exceedingly complex but exciting area of research. My present interests are still with the parathyroids. It seems to me that parathyroids actually have two distinct actions in bone, in that parathyroid influence on bone remodeling through osteoclasts is a distinct and separate action from the control by the hormone of extracellular calcium levels. That is where I am today.

YOUNG: I am in the Department of Anatomy at UCLA. My interest in bone began when I was working part-time in college with a physical anthropologist, Dr. Stanley Garn, who gave me some lateral skull roentgenograms to measure. That led me to Columbia University, where my graduate adviser, Dr. Mel Moss, was working on cranial bones. Ultimately, I became interested in bone in general, and the cells of bone in particular.

MACDONALD: My interest in bone started out with the problem of contamination with radioisotopes some years ago. From then on, we have tried to put some of this knowledge, the metabolism of isotopes, to beneficial use. I would like to learn of some of the advances going on now.

NICHOLS: I am an internist who has quite recently started trying to organize the medical service at the Cambridge City Hospital into a teaching service for Harvard University—a new venture for all three participants. My interests in bone, like Dr. Raisz's, grew from the frustrations of a clinician. I found that there were a lot of questions posed by my patients' problems which simply had no answers. One question I particularly wanted answered was why in uremia one had to give far more sodium chloride by vein to repair hyponatremia than was indicated by all methods of calculation. This indicated to me there must be a reservoir. I went all over Boston and New York trying unsuccessfully to find someone who would let me work on this problem.

FREMONT-SMITH: A reservoir of what?

NICHOLS: Of sodium out of solution somewhere in the body, that sopped up the sodium I was pouring in. Finally, I came back to the man from whom I had originally sought advice, Dr. James Gamble, who said, "Well, you could come here to work." Two years later I left and spent 2 of the most pleasant and productive years of my life. During those years I landed in this field. I started with the idea that the only way to approach the problem was to use an artificial kidney, so I built one. We began dialyzing animals against low sodium baths and analyzing all sorts of tissues using methods taught us by Ernie Cotlove. One of our colleagues in the laboratory was Bill Bergstrom whose work you may know. He worked on methods for the analysis of sodium in bone and analyzed the bones of our animals while we analyzed muscle, skin, tendon, liver, and so forth. Bill Bergstrom found the reservoir and we learned from him how to measure sodium in bone mineral. After a period of studying the effects of sodium loads and depletion on bone mineral composition, I found that other things such as hormones seemed to control the constitution of bone mineral. These obviously had to affect bone cells, rather than bone mineral per se. This view led to work we are doing now, which is a study of bone cell physiology.

FREMONT-SMITH: When you spoke of a reservoir, did you mean an empty reservoir that would fill up with sodium, rather than a reservoir of extra sodium already lying there?

NICHOLS: It was one that could fill and empty.

FREMONT-SMITH: You mean it would fill when you gave sodium to the empty reservoir?

NICHOLS: Right, or vice versa. Now, as an internist, I am back examining people, and asking, when they have metabolic bone disease, how their bone cells behave. I know that I have not found any answers. Indeed, I am considering giving up work on human bone and going back to trying to learn more about the basic cell systems in animal bone so that the human bone problem can be approached more effectively than we are doing now.

FREMONT-SMITH: Dr. Urist, what are your special interests in bone?

URIST: I am Director of the Bone Research Laboratory at UCLA and a practicing orthopedic surgeon. I am interested in biology, medicine, and evolution of the musculoskeletal system. I am interested in the problem of the chemistry of calcification and bone induction, but I am also working on the chondrosteans who had bone in the endoskeleton and, for some unexplained reason, lost it in the bewildering jungle of species in the fossil record of vertebrate evolution.

Dr. Fremont-Smith, before we adjourn this session, do you have any final comments?

FREMONT-SMITH: The only thing I would like to add is that it is quite interesting to listen to the various resources available to our group. Now we have only glimpses of this great variety of different viewpoints and different attitudes, but one of our privileges at this kind of conference is to proceed to tap these resources and to share as much as we can. Thank you.





## HOMEOSTASIS OF CALCIUM

**Discussion Leader:**

**DR. D. HAROLD COPP**

**COPP:** The first point to consider in homeostasis of calcium is the remarkable constancy of the level of calcium in plasma and body fluids in the normal animal. This is illustrated in figure 1, which shows the

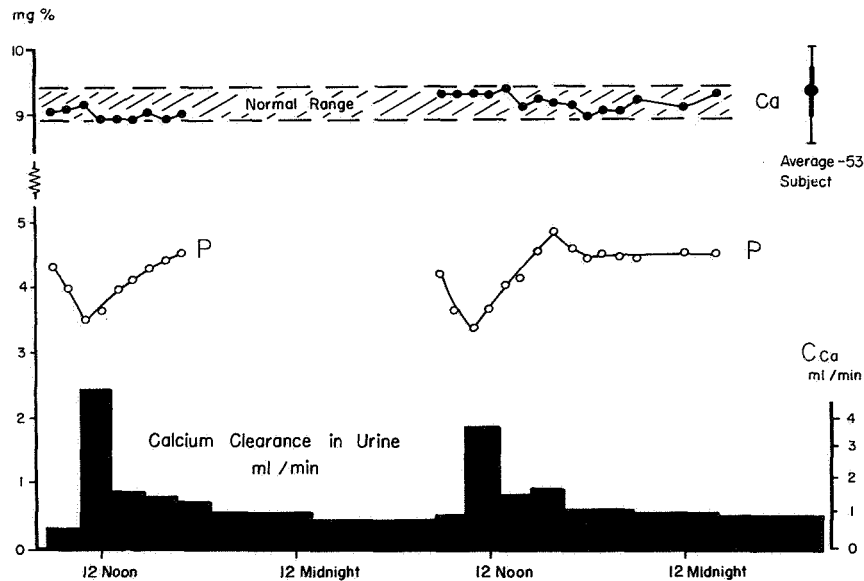


FIGURE 1. Diurnal fluctuations in plasma calcium and phosphorus levels in a normal adult male.

diurnal fluctuations of plasma calcium and phosphorus in a normal adult male subject in Shaughnessy Hospital, Vancouver, British Columbia. Although the calcium level is very constant, there is considerable fluctuation in the plasma phosphate and in the urinary excretion of both elements. The same constancy in plasma calcium has been observed in normal dogs and rats.

The main factors affecting plasma calcium are shown in figure 2 (ref. 11). These factors include absorption of calcium from food

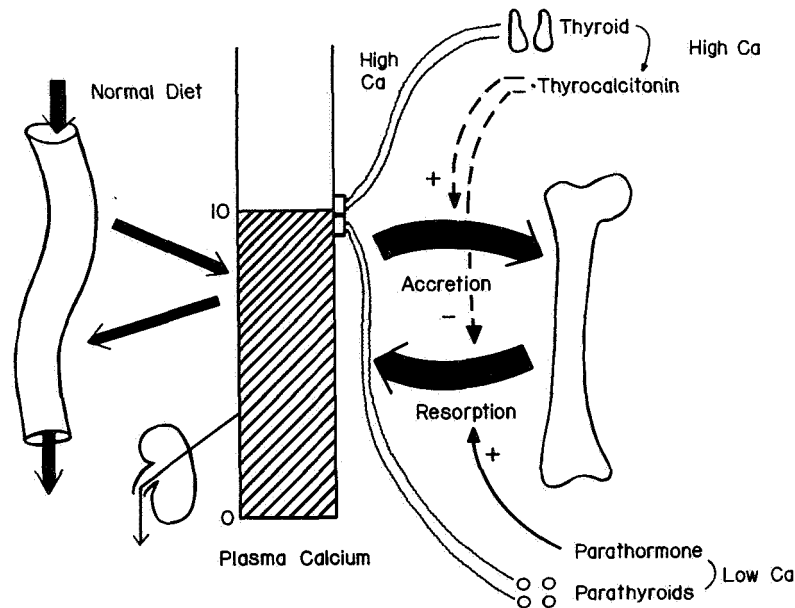


FIGURE 2. Factors involved in control of the plasma calcium level. [Adapted from ref. 11; reprinted by permission of the publisher.]

and excretion of calcium in feces, urine, and sweat. However, the most important single factor is the tremendous reservoir of calcium in bone, which may amount to over 1 kilogram in a 70-kilogram man. We believe that the tiny crystals of bone salt act like the particles of an ion exchange resin, providing a labile exchangeable calcium pool of 3 to 6 grams in a normal adult man, and that this acts as a buffer to help prevent wide fluctuations in the level of plasma calcium. However, in the long run, the balance between calcium accretion in bone formation and calcium release from bone during osteolysis is more important. I believe that these two processes are affected by the concentrations of calcium and phosphate in blood, by the presence or absence of vitamin D, and particularly by the activity of two potent hormones—the parathyroid hormone, produced by the parathyroids, and calcitonin, or thyrocalcitonin, from the thyroid. Diet is also a factor. Figure 3 shows that normal rats fed a phosphate-deficient diet are hypercalcemic in the morning.

RAISZ: Why do you think they are hypercalcemic in the morning?

COPP: Rats feed during the night, and increased metabolic activity requires phosphate for phosphorylative reactions. This increased activity, particularly on a phosphate-deficient diet, lowers the plasma phosphate, and in my opinion this result interferes with bone deposition of calcium. At the same time, calcium is absorbed from the gut at night.

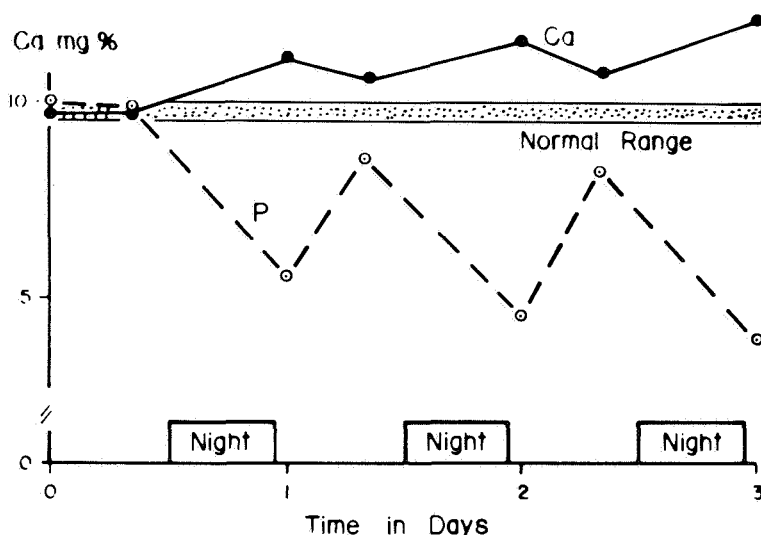


FIGURE 3. Changes in plasma calcium and phosphate in 5-week-old rats restricted to a diet low in calcium and phosphate. Note the marked changes in plasma phosphate associated with nocturnal feeding habits.

FREMONT-SMITH: Is most of the food intake at night?

COPP: Yes. Rats eat almost entirely at night. If you turn off the lights in the daytime you may be able to persuade them to eat something, but rats are rather shy and carry on most of their activities at night.

HOWELL: Do you have similar data for man?

COPP: No; we do not. It is almost impossible to get a phosphate-free diet that is acceptable to man.

NICHOLS: It is an important point then that this cannot be observed in man except to the degree that one can restrict the availability of dietary phosphate.

COPP: You may get some response in a hyperparathyroid patient with hypercalcemia when phosphate restriction may increase the plasma calcium level.

Our rats had essentially no phosphate in their diet and were growing rapidly, so that the phosphate required for soft tissues was obtained at the expense of the skeleton. We have shown that parathyroidectomized rats fed a low phosphate diet will develop hypercalcemia, even when the diet is also low in calcium.

PRITCHARD: I would like to ask a question in reference to what you have said. In reading about calcium and phosphate concentrations, one finds that one level always seems to go up as the other concentration goes down. Do calcium and phosphate always move in opposite directions?

COPP: Sometimes changes in calcium and phosphate move in the same direction, sometimes opposite. It depends on many factors.

PRITCHARD: I thought you were assuming that because the phosphate was down the calcium must go up.

COPP: No. With these phosphate-deficient diets we find that as blood phosphate falls, blood calcium rises. This is true particularly in the parathyroidectomized rat.

PRITCHARD: Is this a matter of mass action, or is it something biologic?

COPP: I think there may be two factors. Low concentrations of phosphate may affect the rate of bone deposition of calcium. At very low blood phosphate concentrations, there may be little or no formation of new bone mineral; high concentrations may have the opposite effect. This would account for a reciprocal relationship between calcium and phosphate and would account for some of the observations of Fuller Albright. I feel that an effect on bone formation would be critical. Phosphate deficiency also affects the response of the animal to parathyroid hormone and thyrocalcitonin. There will also be an effect on calcium absorption from the gut; this absorption is enhanced at low phosphate concentrations and reduced when the dietary phosphate is high. We observed that phosphate deficiency with low plasma phosphate also results in tremendous urinary excretion of calcium (ref. 12).

PRITCHARD: It is doubtless not a simple relationship.

COPP: That is true.

BAUER: In general, Dr. Copp, do your data show that your animals had enough vitamin D?

COPP: Yes. The animals received adequate but not excessive vitamin D. The diet contained 20 U.S.P. units vitamin D per gram, which is 7 times the recommended minimum.

I will assume that we all agree that plasma calcium is controlled with remarkable precision and efficiency in the normal animal. Figure 4 (ref. 13) shows that this precise control is lost when the thyroid and parathyroid glands are removed. In 200 normal dogs, the fasting plasma calcium concentration falls within a rather narrow distribution curve. In the thyroparathyroidectomized dogs, there is a great variability in the fasting plasma calcium concentration. This impairment in homeostatic control is demonstrated even more dramatically when the system is stressed by infusion of calcium or of the calcium chelating agent, EDTA (ethylenediaminetetraacetate). Sanderson et al. (ref. 14) found, in normal dogs (fig. 5), that the plasma calcium returned to the original concentration a few hours after it had been raised by infusion of calcium or lowered by injection of EDTA. This precise control was lost after thyroparathyroidectomy, as shown in figure 6, and the blood calcium was still not back to the original concentration

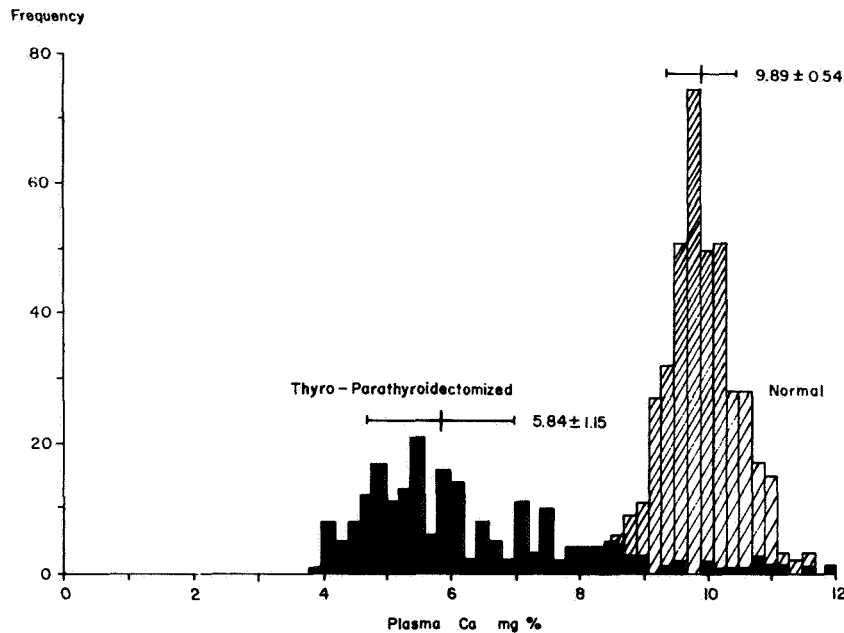


FIGURE 4. Distribution of fasting plasma calcium concentrations in normal and thyro-parathyroidectomized adult dogs. [Adapted from ref. 13; reprinted by permission of the publisher.]

24 hours later. It is apparent that the glands were required for rapid, efficient homeostatic control of both hypocalcemia and hypercalcemia.

FREMONT-SMITH: There is a tendency to return toward normal values that starts quite promptly, is there not?

COPP: Yes. This tendency to return toward normal was observed even in the thyro-parathyroidectomized dogs, but it occurred much more slowly.

FREMONT-SMITH: The reason I raise this point is because L. J. Henderson found, in his work on the constancy of the pH in the blood (ref. 15), that as one homeostatic factor after another was removed, secondary factors came in. I wonder whether there were not secondary factors to manage the calcium concentration in this situation.

COPP: Yes, I think so. There is a gradual recovery to the initial concentration within 24 to 48 hours following induced hypocalcemia or hypercalcemia. I believe that this is a result of a secondary and less sensitive control. If we assume that the rate of calcium deposition in bone is a function of the ionic calcium concentration, hypercalcemia should increase calcium deposition in bone and lower plasma calcium; yet hypocalcemia will have the opposite effect.

FREMONT-SMITH: Is the bone reservoir the main secondary factor?

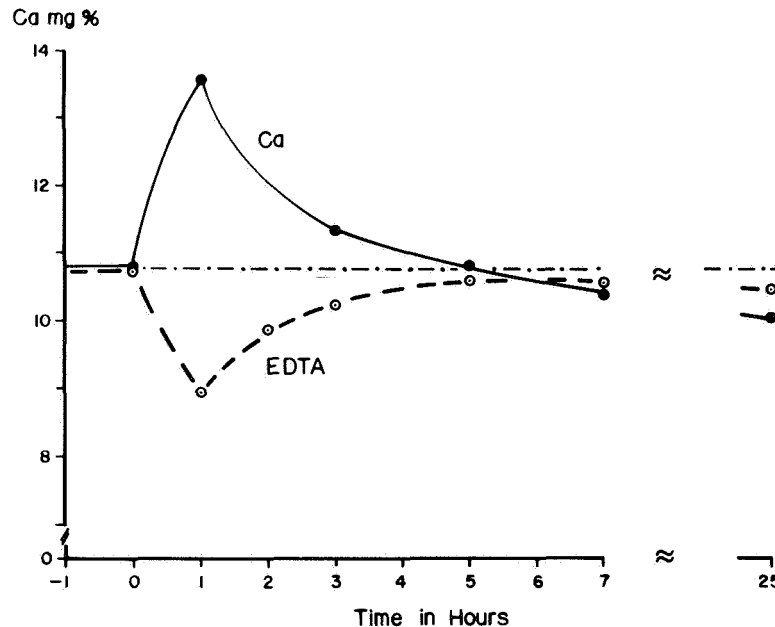


FIGURE 5. Changes in plasma calcium during and after intravenous infusion of calcium (15 mg/kg) or the EDTA equivalent of calcium (5 mg/kg) for 1 hour in normal adult dogs. [Replotted from data of Sanderson et al., ref. 14.]

COPP: Yes, and the relative balance between accretion and resorption.

PECK: What is the effect of age on this process?

COPP: Recovery is much faster in young animals than in old ones, not only with regard to the hormone response in intact animals, but also with regard to recovery in parathyroidectomized dogs.

HOWELL: In the dog, is there not a problem of accessory gland remaining in the mediastinum?

COPP: We do not worry about this. Our animals behave as if they were parathyroidectomized, and in over 200 operations we have seen no evidence of functional accessory glands.

TALMAGE: I would like to stress briefly the homeostatic control of calcium in the absence of the parathyroid gland. Even in the absence of the gland, plasma calcium concentrations tend to equilibrate, although at a lower concentration. However, because the equilibration process under these conditions is based primarily on physicochemical properties, the rate of equilibration is much slower. On a calcium-free diet, a basic level will be maintained and will be affected only by the low rate of calcium reabsorption by the kidney tubule. If an animal is maintained on a high calcium diet, the basic level may never be reached; the calcium concentration in the blood may be extremely

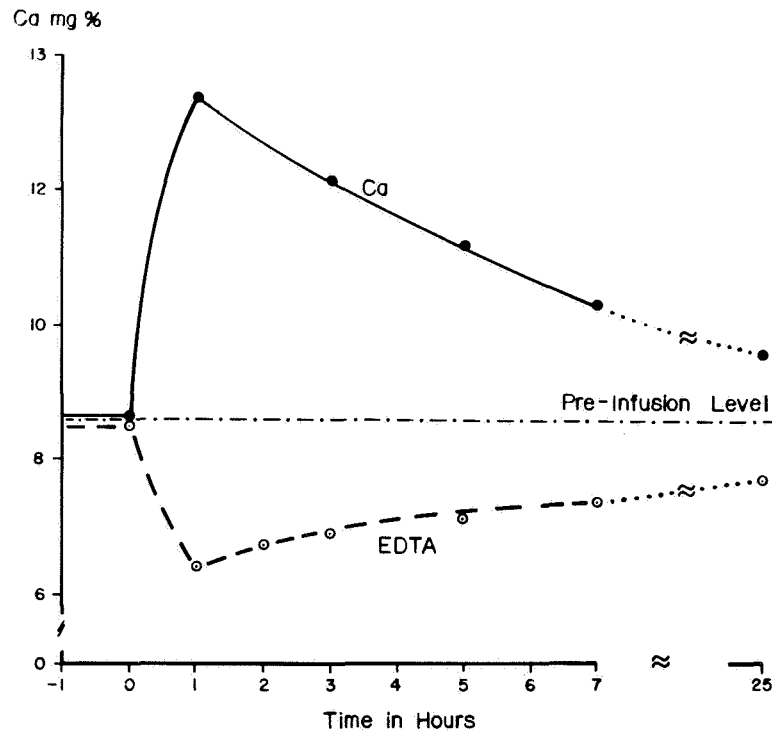


FIGURE 6. Changes in plasma calcium during and after intravenous infusion of calcium (15 mg/kg) or the EDTA equivalent of calcium (5 mg/kg) into thyro-parathyroidectomized dogs. Note the impaired homeostatic control of both hypocalcemia and hypercalcemia. [Replotted from data of Sanderson et al., ref. 14.]

variable and may even reach a concentration higher than that seen in the normal animal. The important point is that even in a parathyroidectomized animal the bone can provide calcium to the fluid compartments.

COPP: This is one of the secondary factors that Dr. Fremont-Smith mentioned.

WALSER: I would like to raise a slightly semantic objection which I raised some years ago. EDTA does not induce hypocalcemia; it induces hypercalcemia. The same is true of citrate or protein. You can raise plasma calcium by infusing either. I do not think you would use the word *hypocalcemia* for those situations and I do not see why we should for this situation.

COPP: Your semantic point is well taken, but it does not alter the fact that EDTA produces physiologic hypocalcemia.

WALSER: I think the assumption that physiologic calcium has been measured by what you have illustrated is a little risky.

COPP: I do not agree. This is evident from the response of the parathyroids to EDTA-induced hypocalcemia.

RAISZ: I would like to carry this further. A question pertinent to your study is whether there is any effect of EDTA separate from the chelating of calcium. I agree that ionized calcium effects probably determine the responses in the whole animal where a modest amount of EDTA is being infused. When larger amounts of EDTA are given, however, we are no longer studying the effects on calcium but studying the effects of EDTA on tissue which can be substantial. EDTA has toxic effects on transport systems of cell membranes in many tissues that we have studied; these effects are entirely separate from the calcium effect.

COPP: I agree completely. EDTA complexes a number of important trace metals and prolonged administration may have deleterious effects. However, I do not think that these effects are important if you infuse EDTA for only a few hours, as has often been done in human subjects. But, for example, after 2 to 3 days of EDTA infusion in sheep, the animals often sickened and died.

TALMAGE: One must keep in mind that the same type of response may be obtained without EDTA. This response can be found in any method by which plasma calcium is lowered dramatically. The calcium invariably returns rapidly to the predetermined concentration.

NICHOLS: I do not think that EDTA's effect on tissues should be discussed as necessarily being unrelated to calcium. Indeed, it is probable that the transport effects you observe are actually effects on the membrane structure, which in turn are probably related to the binding of calcium into these membranes. For example, one can abolish sodium and potassium transport in red cells with EDTA and restore it again by adding enough calcium to saturate the EDTA present and leave a small excess (G. Nichols, unpublished observations).

RAISZ: But you can show that EDTA is bound to the membrane itself; that is, that even after all the calcium has been chelated there is still further binding of EDTA. Another point is that such ions as citrate, oxalate, or phosphate can be used to lower the calcium. We have had better results with these than with EDTA in the whole animal (ref. 16).

NICHOLS: I would like to comment about man and EDTA infusions. One can obtain data in man, which are not dissimilar from yours, Dr. Copp, as probably many here have done. Our own experience with man has been a fair copy of what you have shown in animals, with the exception that the duration of the whole phenomenon seems to be quite a bit longer. This is probably partly because we have infused for 4 hours rather than for 1 hour (with the specific intent of being sure the parathyroids have been shut off), but we do not get calcium returning to normal following EDTA infusion for quite a few hours. Incidentally, the same is true for calcium infusions in man.



COPP: This probably reflects differences in the metabolic activity of the skeleton.

ARNAUD: Dr. Nichols, are these data from a parathyroidectomized subject?

NICHOLS: No. The data were from normal people. If you have a parathyroidectomized subject the response is even slower. Another interesting phenomenon is that in carcinoma of the parathyroid, or occasionally in adenoma with hyperparathyroidism, serum calcium concentrations may go up and stay up after calcium infusions. We have seen them still elevated 4 days later.

COPP: The next two figures illustrate this point. Figure 7 shows the plasma calcium curve for a normal adult human male subject following a 1-hour calcium infusion. Recovery is somewhat slower than in the dog, but after 24 hours plasma calcium concentration is almost back to normal. Figure 8 shows the curve following a similar infusion in a thyroparathyroidectomized adult human male subject of similar age, and confirms Dr. Nichols' observation that the calcium concentration in plasma remains elevated.

NICHOLS: Dr. Talmage points out that this is probably related to the fact that an adult man does not use his gut as a calcium pool for

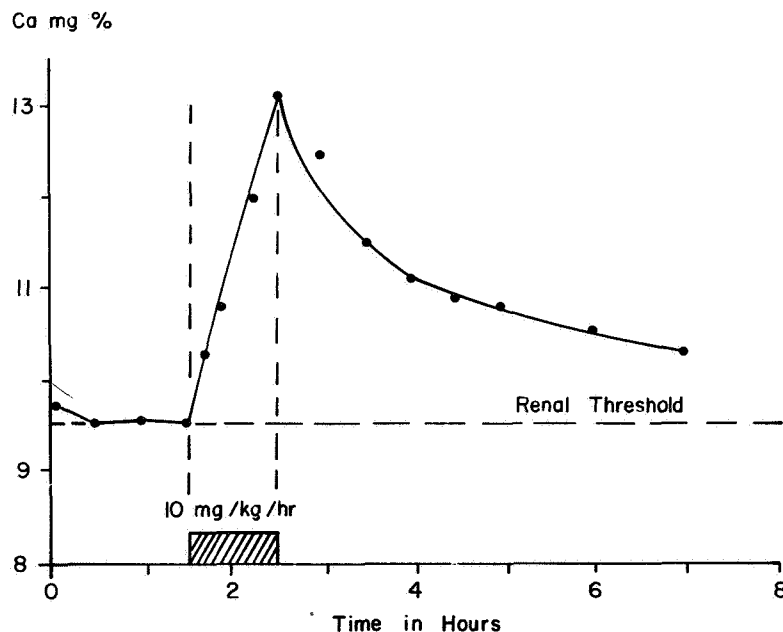


FIGURE 7. Changes in plasma calcium during and after intravenous infusion of calcium (10 mg/kg) in a normal adult man. [From the Clinical Investigation Unit, Shaughnessy Hospital, Vancouver, B.C.]

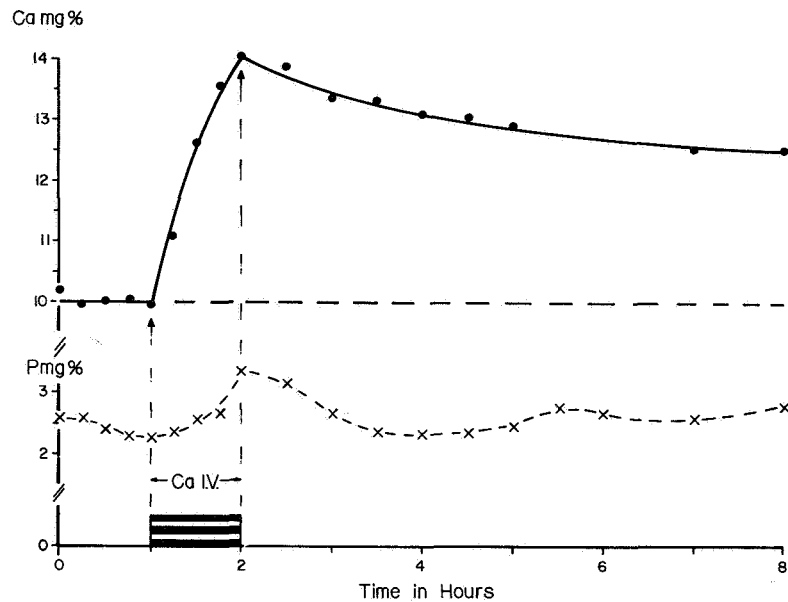


FIGURE 8. Changes in plasma calcium during and after intravenous infusion of calcium (10 mg/kg) into an adult man who had been thyroparathyroidectomized a number of years earlier. Note the impaired control of hypercalcemia. [From data supplied by Dr. H. W. McIntosh, Department of Medicine, University of British Columbia, Vancouver, B.C.]

ready availability but instead depends upon his bone which is kept in a state of availability by the presence of parathyroid hormone.

HOLTZER: How did this patient have a normal calcium concentration in the beginning? Was he on vitamin D therapy?

COPP: He was maintained on a high calcium diet of 0.2 milligram thyroxine and 50 000 IU vitamin D daily.

The second item to be considered in our discussion should be the function of bone in calcium homeostasis. Over 99 percent of the calcium in the body is located in the skeleton, and this amount may be over 1 kilogram calcium in a 70-kilogram man, as mentioned previously. It is also probable that the regulating hormones from the thyroid and the parathyroid glands exert their effect primarily by action on bone. Considering the nature of bone mineral, we find the most significant physical feature is the extremely minute dimensions of the crystals, which provide an enormous area for surface exchange. In many respects these crystals are comparable to the resin particles of an ion exchange column and act in much the same way to release or to adsorb ions present in the surrounding body fluids. Figure 9 shows a stylized model of such a crystal and indicates the way in which calcium on its

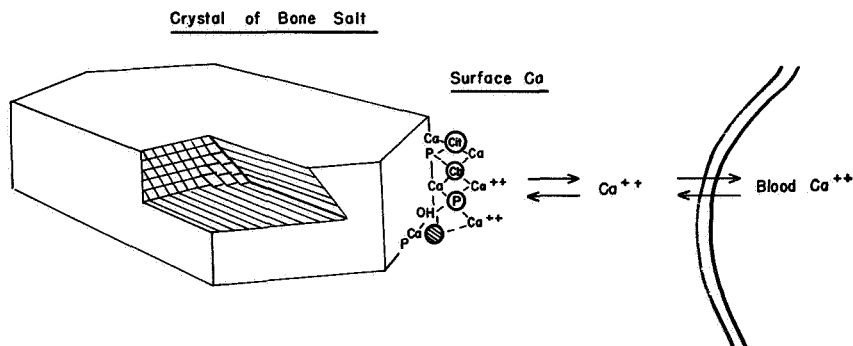


FIGURE 9. Diagrammatic model of exchange between calcium in blood and calcium on the surface of the crystals of bone mineral.

surface could exchange with calcium in plasma. In my opinion, this surface exchange is a physicochemical process that is independent of the energy-dependent and biologically active processes of accretion and bone resorption.

Dr. Robinson, will you comment on this and on the ultrastructure of bone where these processes probably take place?

ROBINSON: The crystal in figure 9 is one which I think is perfectly valid. With better resolution we still observe that those bone crystals large enough for their habit to be observed are tablet shaped. According to X-ray and spot electron diffraction patterns their fundamental atomic lattice pattern is that of hydroxyapatite with some variability of their simple surface composition. These small crystals are probably flattened plaques, which increases the surface-to-mass ratio; these crystals are supposed to occupy, as we conceive the process in mineralization, water space that originally existed in the matrix shortly after this matrix was formed by the cell.

As the crystals occupy this space and the water is displaced to give them room, less and less water becomes available for ion exchange in areas around the surface of these crystals. Finally, the situation is such that in a fully mineralized bone matrix, the crystals on the periphery of the mineralized matrix masses are the only ones in actual continuity with extracellular fluids free enough to allow ion transfer.

PRITCHARD: Dr. Robinson, how big are those mineralized matrix masses? What should be one's picture of a mineralized mass of crystals, and are they really crystals?

ROBINSON: Yes; I believe they are crystals.

PRITCHARD: Is that mass the whole mass between one blood vessel and the next?

ROBINSON: No; this is extremely variable. In some areas in bone the matrix is very poorly mineralized and in other areas the matrix is rather compact. I will discuss this subject in more detail later.

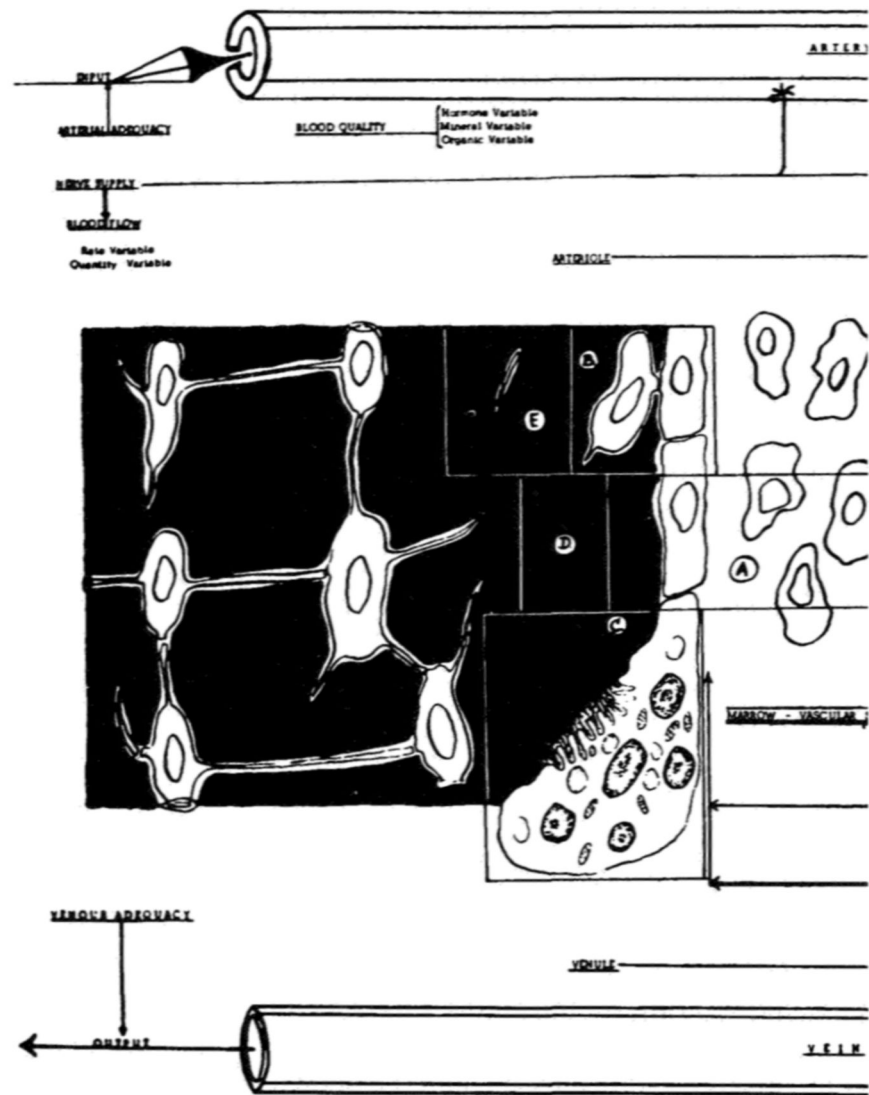
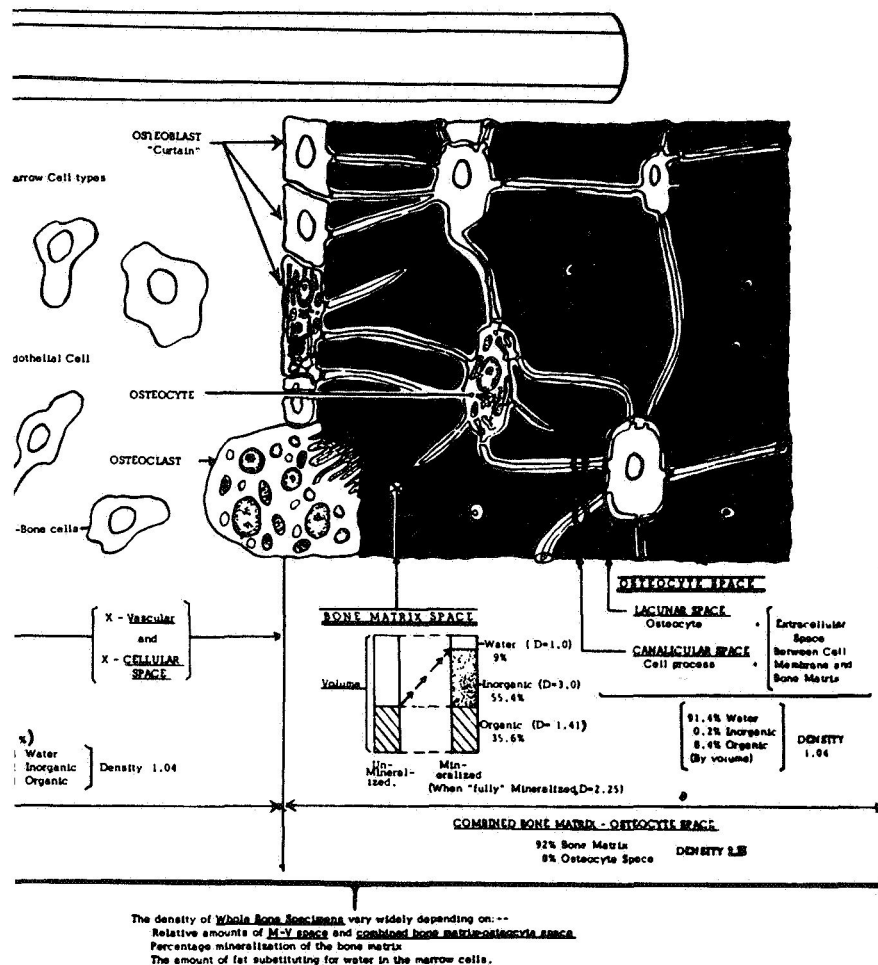


FIGURE 10. Bone as a physiologic unit. [From re

Figure 10 is a diagram illustrating the basic concept of the physiologic unit of bone (refs. 17 and 18). This concept shows the influx of blood and the outflow of blood; there are endothelial cells with a basement membrane which, incidentally, forms the container for the blood as it goes through the bone. Between the surfaces of these endothelial cells and the mineralized matrix, one can occasionally see



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polymorphonuclear cells and a variable number of other cells. (The electron-microscope studies reported here were supported by Public Health Service, National Institutes of Health (NIH) research grant no. AM00706 and NIH training grant in orthopedic surgery no. TI AM 5317.)

In some places, particularly on the surface of newly forming bone

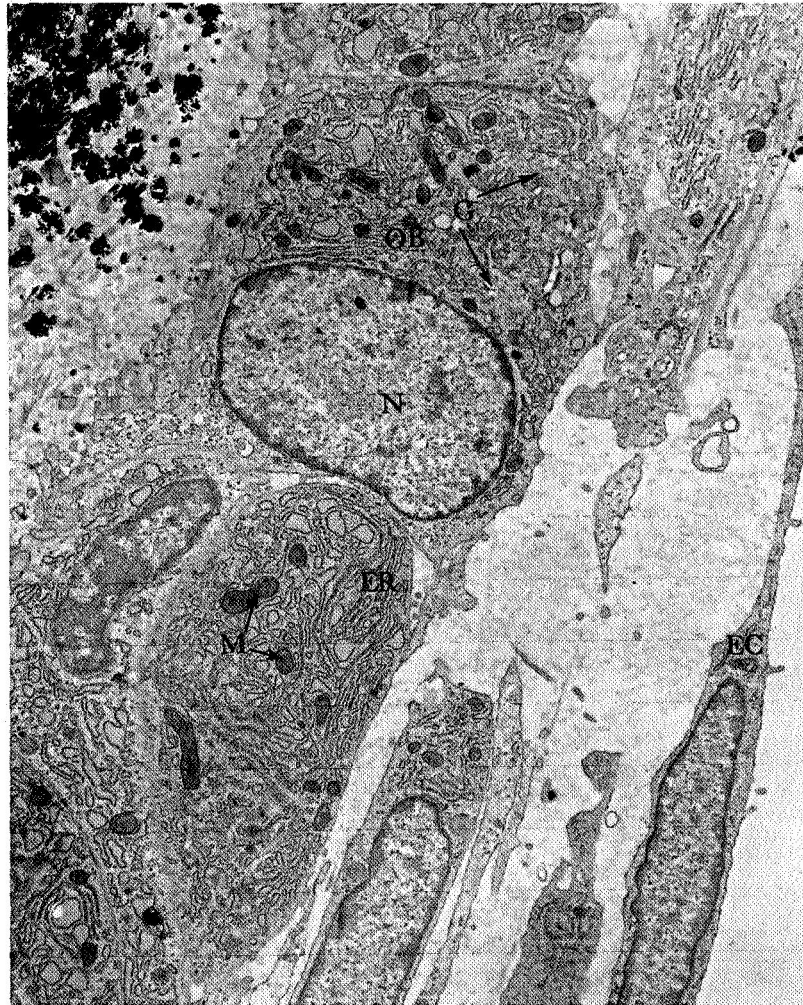


FIGURE 11. An electron micrograph of the surface of metaphyseal trabecular bone from a young growing rat. On the far right lower two-thirds of the figure is a vascular lumen. Then, proceeding to the left upper corner, one observes an endothelial cell (*EC*), several intermediate cells among which are probably some preosteoblasts, and then a layer of closely packed osteoblasts (*OB*). The osteoblast cytoplasm contains a well-developed rough endoplasmic system of membranes (*ER*), mitochondria (*M*), a juxtanuclear Golgi apparatus (*G*), and a nucleus (*N*). The osteoblasts in this situation appear to form a curtain over the underlying bone matrix. In the left upper corner, one observes the very incompletely (spottily) mineralized collagenous bone matrix. However, as one can observe in figure 12, the mineralization becomes quite complete in this type of specimen within a few hundred angstroms of the collagenous periphery of the bone-matrix mass. Approximately 6780 $\times$ . [Reprinted by permission of Dr. David Cameron, University of Sydney, Sydney, Australia.]

on metaphyseal trabeculae (figs. 11 and 12) and along the walls of vascular canals in osteons that are actively filling (fig. 13), osteoblasts form a closely packed, continuous layer of cells over the bone matrix. However, in regions of longer standing bone matrix, the osteoblasts,

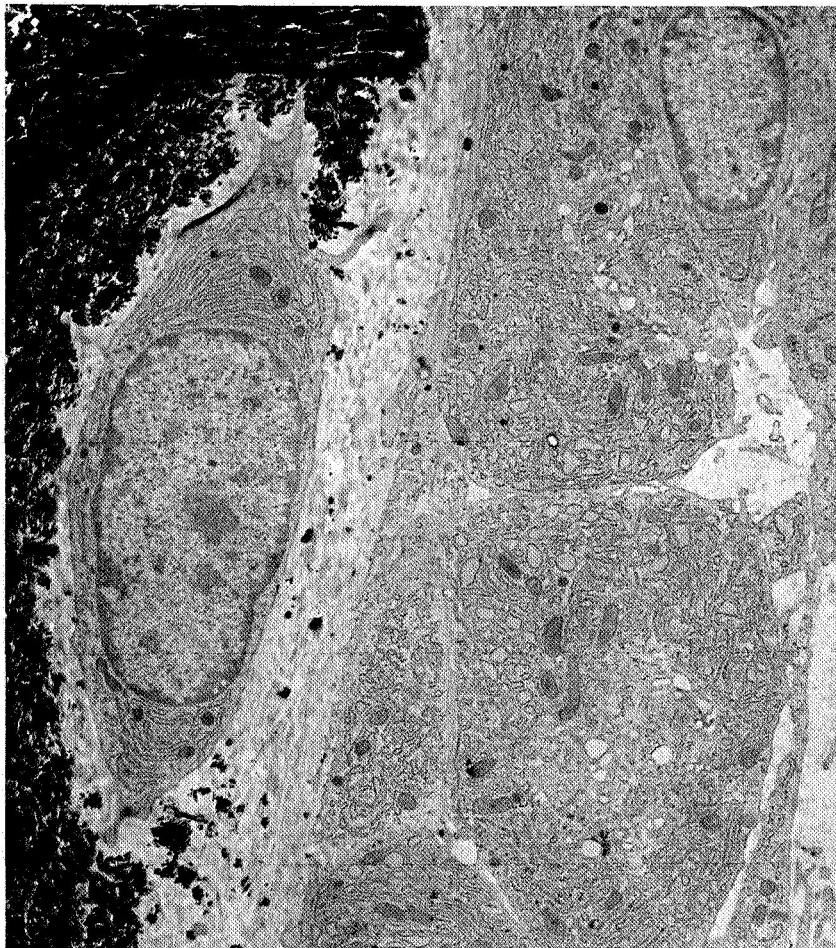


FIGURE 12. The topography and magnification of this figure is similar to that of figure 11. However, in this micrograph one can observe an osteoblast becoming surrounded by bone matrix. As noted in the text, there are some round, dense masses of detritus overlying the cells and matrix which are probably small contaminating deposits of lead acetate. On close inspection these can be distinguished from the rough clusters of bone crystals forming spottily in the generally unmineralized bone matrix surrounding the surface osteocyte and underlying the curtain of four or five osteoblasts. The more fully mineralized bone matrix can be seen in the left upper corner about 1 micron from the nucleus of the osteocyte. Approximately 6780 $\times$ . [Reprinted by permission of Dr. David Cameron, University of Sydney, Sydney, Australia.]

or bone-matrix-covering cells, may be strung out in a tenuous cell cover over this matrix. In electron micrographs of haversian canals in midfemoral cortex of adult dogs, there appear to be spaces between or fenestrations through these bone-matrix-covering cells; one would think these spaces might allow unobstructed contact of extracellu-

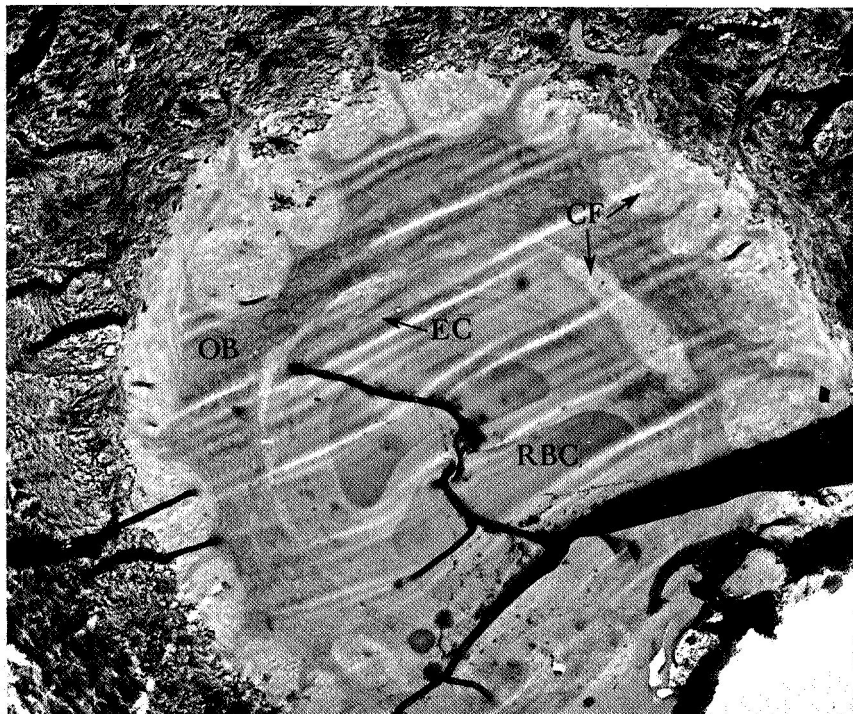


FIGURE 13. Electron micrograph of a cross section of a haversian canal from the mid-diaphyseal cortex of the femur of a puppy. The dark black lines and white streaks are wrinkles in the section. Near the center are two red blood cells (*RBC*) in the lumen of the haversian capillary. The capillary is usually surrounded by two or three endothelial cells (*EC*). They have a basement membrane. The intermediate cell (*IC*) is not further described because it could be a preosteoblast, a pericyte, or an extension of an overlapping endothelial cell. Particularly in canals in which osteoblasts (*OB*) appear to be in an active phase of protein synthesis as do those in this figure, one or more of these intermediate cells are seen.

Collagen fibrils (*CF*) in this specimen (which was fixed in glutaraldehyde, embedded in Epon, and counterstained with a lead acetate solution) appear as white disks in a more dense (darker staining) cement substance. Also note that unlike the situation at the periphery of trabecular bone, bundles of collagen fibrils appear between the vascular space and the osteoblast curtain; occasionally mineralization is observed in these regions as well as in the bone matrix placed centrifugally to the osteoblasts. Approximately 3570 $\times$ . [Reprinted by permission of Dr. Reginald Cooper, University of Iowa Medical School.]



lar fluid with bone matrix. Therefore, the cell cover is morphologically different over the surface of forming and newly formed bone matrix and over the surface of longer standing, quiescent, or resting bone where there is no morphologic evidence of bone resorption or accretion. Of course, *in vivo*, this tenuous cell cover over the surface of a mass of completed and mineralized bone matrix may be a dynamic one with more power over the physical state of the extracellular fluid, which "laps up" on the "shore" of mineralized bone matrix masses, than is suggested by morphologic observations such as these. Such resting osteoblasts may respond in an as yet undelineated manner to the influence of hormones, vitamins, nerves, and chemical and pH changes in their environment.

The ratio of the mass of mineralized bone matrix to the available crystal surfaces on its periphery is difficult to calculate for two reasons: first, the crystals project irregularly into extracellular and free bone matrix fluid along the periphery of a mass of mineralized bone matrix; second, the periphery of even a fully mineralized bone matrix mass may be, for a depth of several hundred angstroms, incompletely mineralized. Therefore, one cannot exactly calculate the surface-to-mass ratio as one could if the surfaces of the mineralized bone matrix masses were smooth and if full mineralization of their bone matrix extended right up to those smooth surfaces. In other words, there is a surface roughness factor and a peripheral depth factor which make surface-to-mass calculations difficult.

I attempted to estimate the apatite crystal surface area which presents on the periphery of the mineralized bone matrix masses in the cortical-type regions of the skeleton in a 70-kilogram man. I concluded that there should be between 1500 and 5000 square meters of bone crystal surface exposed to extracellular fluid on canalicular, lacunar, and vascular (haversian) canal surfaces. The variable factors of roughness and peripheral ion permeability of bone matrix masses tend to make this estimate of lesser magnitude than the true value (ref. 18).

PRITCHARD: I was actually referring to the little units bounded by the canalicules. Presumably these have a volume of the order of a cubic micron and a calculable external surface area. But then, there are the surfaces of the individual crystals inside this mass, and one does not know what pathways exist between these surfaces and the major pathways that you have shown.

ROBINSON: That is correct in terms of direct observation. However, on the basis of many experimental data (refs. 19 and 20), I finally developed a theory (ref. 17). I have chosen to call this the water-bridge theory. It explains the major factor governing the transit of calcium ions to and from the surfaces of individual apatite crystals

in the mineralized bone matrix masses of the skeleton (ref. 21).

The water-bridge theory applies only directly to those ions or molecules that require a certain freedom of water molecules for transit through extravascular and extracellular fluid. For instance, this theory does not apply to noble gases; radon apparently does not need water molecules to move into and out of mineralized bone matrix. In relation to bone, the water-bridge theory is as follows:

1. Bone matrix is formed by osteoblasts. When first synthesized prior to matrix mineralization, the matrix is highly hydrated and does not contain bone crystals. It does, however, per unit volume, contain its full complement of organic solids (ref. 22).

2. As bone matrix mineralizes, the bone crystals displace mainly water, volume for volume, rather than the collagenous organic solids which form the bulk of the organic solids in bone matrix. In cartilage (ref. 23) and possibly in bone (ref. 24) a large part of the non-collagenous protein, associated with the mucopolysaccharide of the original organic matrix, and some sulfated mucopolysaccharide are apparently displaced from any given volume of matrix, during mineralization. Some free fibrous (collagenous) protein molecules that are not incorporated or adequately crosslinked into fibrils may move out of the matrix prior to and during mineralization. However, in normal bone, the collagen solids, per unit volume of bone matrix, are not lost during matrix mineralization.

3. The original volume of bone matrix does not shrink or expand during mineralization.

4. Bone matrix water is not completely displaced during mineralization. At full mineralization, the residual bone matrix water becomes bound and can no longer form a water bridge for the transport of calcium ions. Each calcium ion needs, theoretically, two water molecules for rapid movement. These water molecules must have a degree of freedom not present in residual bone matrix water in fully mineralized bone matrix. This, of course, does not prevent the transit of calcium ions to the surfaces of bone matrix mineralization. It is our hypothesis, for instance, that diffuse exchange, at the light-microscope level of bone organization, in terms of  $^{45}\text{Ca}$  autoradiographs, is actually caused by beta particles emanating from ions of the calcium isotope which have found or are finding their way via extracellular fluid to crystal surfaces on the walls of canaliculi which permeate the fully mineralized bone matrix. The walls of canaliculi are normally fully mineralized right up to the extracellular fluid-mineralized matrix mass interface. Proof or rejection of this hypothesis is being pursued at present by the use of combined electron microscopy and autoradiography (ref. 21).

Part 4 of this theory applies generally to availability of mineral

crystal surfaces or mineral agglomerates and mineral-organic agglomerates other than crystalline, in any organic matrix, and not only in bone, but also in cartilage, and in ectopic sites of ossification and calcification. If there is no free water bridge in a tissue, then the ions and molecules that depend on this bridge for transit cannot move through the tissue to preferred sites of fixation or exchange.

5. Implicit in this theory are the following two corollaries:

a. The density of electrostatic forces progressively increases per unit volume of bone matrix as mineralization increases, limiting the movement through the matrix of particles carrying charges.

b. Surface-adhering forces that are of lesser strength than electrostatic forces and that are active even on particles not carrying a charge will increase per unit volume of bone matrix as crystal and fibrous protein surfaces become crystals on the periphery of a mass of fully mineralized bone matrix.

Conversely, when bone matrix mineralization is quite incomplete, e.g., when it contains one-half or one-tenth as much bone mineral per unit volume as it can contain when fully mineralized, then a water bridge of a sufficient number of free water molecules is present for the rapid transit of calcium ions to the surfaces of most of the bone crystals in the whole mass of quite incompletely mineralized bone matrix. If this volume of quite incompletely mineralized matrix is considerable in any one region of the skeleton, a hotspot (in terms of autoradiographs viewed at the light-microscope level of tissue organization) is present, and a region of low microradiographic density is present.

In the case of the periphery of fully mineralized bone matrix masses, the surface consists of bone crystals and possibly some noncrystalline mineral agglomerates, the surfaces of which are in continuity with extracellular fluid. The water molecules of the extracellular fluid have sufficient freedom to act as a rapid transport device for those ions and molecules which need free water molecules for movement.

The periphery of a fully mineralized mass of bone matrix may be more or less incompletely mineralized for a varying depth. The amount of calcium isotope picked up on the periphery of an otherwise fully mineralized matrix mass will be dependent on the depth of the peripheral zone of incomplete mineralization and is progressively more closely approximated as full mineralization is approached.

These forces—the electrostatic and surface-adhering forces—are conceived of as halting the rapid movement of all particles, including residual matrix water molecules, as bone matrix achieves full mineralization.

The concept of the mineralized mass is best shown in relation to

some work by Cooper et al. (ref. 25) on the haversian canals and their structure. The cortical or compact bone of the long bones of man, for example, is a major contributor to the total bone mass in the whole body. Because there are so many square meters of crystal surface on the periphery of fully mineralized bone matrix masses in cortical bone which face on intracortical vascular canals, canaliculi, and lacunae, one hesitates to regard this vast crystal surface-extracellular fluid interface as an unimportant part of the mineral homeostatic mechanism. Osteoblasts and osteoclasts are in close proximity to this interface and are certainly implicated in everything that takes place there.

I am concerned that metaphyseal bone, particularly that portion lying on the periphery of metaphyseal trabeculae, is usually considered to play the predominant role in mineral metabolism, when the cortical bone forms a much greater portion of the total bone mass in the skeleton and presents an extensive area of bone crystal surface to extracellular fluid in close association with bone cells. These morphologic factors, as I see it, make cortical-type bone more important than trabecular-type bone in total mineral homeostasis.

Figures 13 to 16 show electron micrographs of intracortical vascular (haversian) canals, canaliculi, and lacunae obtained from the mid-cortex of the femoral diaphysis of puppies and adult dogs.

Figure 13 is a cross section of an actively filling-in haversian canal from a puppy. One observes the blood vessel composed of two or three endothelial cells. Peripheral to this vessel along the bone matrix wall of the canal are several osteoblasts. One can observe the protoplasmic extensions from these osteoblasts extending through canaliculi into the bone matrix. These are particularly well seen in figure 14.

The little bone crystals are located in the region of mineralized bone matrix. Between the osteoblasts and the mineralized portion of the bone matrix is a zone of nonmineralized matrix. More peripherally, one observes mineralization of the matrix. This initial mineralization is spotty and is localized in and about collagen fibrils. A few hundred angstroms deeper into the bone matrix the mineralization is more complete, and crystals obliterate the images of collagen fibrils and the space between the fibrils. There are about 5000 to 10 000 angstroms of matrix space between the surface of the lining cells of this part of this haversian canal and the more fully mineralized portion of the bone matrix mass.

The peripheral zone of nonmineralized and partly mineralized bone matrix is highly hydrated. In the zone where mineralization of the bone matrix begins, the hydration starts to diminish and the crystal population starts to increase without any expansion or con-

traction of the original unmineralized volume of this matrix. As the mineralization becomes more and more complete, the residual matrix water would, according to the water bridge theory, be progressively more bound and decreased in amount per unit volume of

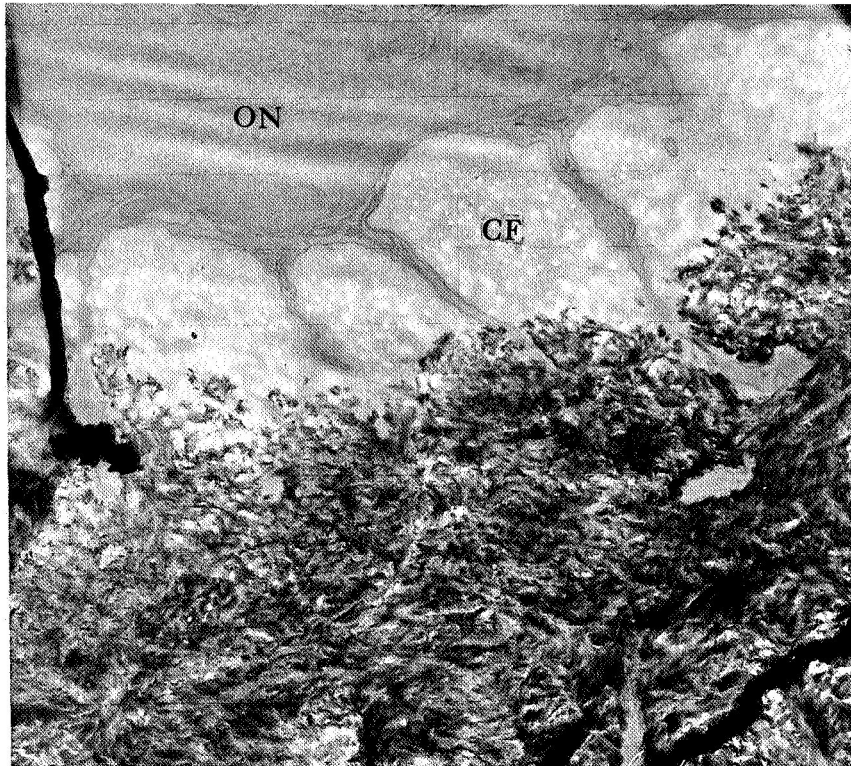


FIGURE 14. Electron micrograph in which the osteoblast-bone matrix interface at the periphery of a haversian canal is illustrated. At the top an osteoblast nucleus (*ON*) predominates the scene. A thin zone of osteoblast cytoplasm with fingerlike projections borders the nucleus. The cytoplasmic projections covered by plasma membrane are observed to extend through lacunae into the bone matrix. The bone matrix is unmineralized close to the osteoblast, and the collagen fibrils (*CF*) cut transverse to their long axes are seen as circular zones in a more densely staining ground substance which contains Epon and was counterstained with lead acetate. The mineralization of the bone matrix becomes more complete as one moves farther away from the cell. The roughness of the mineralized periphery of the calcified matrix mass, represented by bone crystal clusters, and the depth factor, represented by bone matrix in which incomplete mineralization is present on the periphery of the mineralized bone matrix mass, are both obvious in this picture. In regions of newly mineralizing matrix it would appear that (in this instance under very high magnification) the cement substance between the fibrils was mineralizing prior to fibril mineralization. Many exceedingly small crystals can be seen in this region. Approximately 18 000  $\times$ .

bone matrix, and thereby prevent any rapid exchange of calcium ions between those ions on crystal surfaces in the more fully mineralized region and those ions in the extracellular fluid in the unmineralized matrix region at the periphery of the haversian canal.

There are a good many artifactual cracks in the mineralized matrix in our early specimens, but these cracks have been eliminated in many later sections of fully mineralized cortical bone by formulating the embedding medium used to infiltrate the haversian canals so that it equalizes any strain between itself and the surrounding fully mineralized bone matrix.

One can see that the surface of a mineralized bone matrix mass, in terms of bone crystal surfaces, is anything but smooth, and that the phase change from nonmineralized to fully mineralized bone matrix may be a gradual one giving depth to the periphery of the mineralized bone matrix mass.

BÉLANGER: Could I make a point here, Dr. Robinson? I think one thing that confuses people when they are talking at the same time of electron micrographs and microradiographs is that microradiographs are generally first image pictures, otherwise called a negative, whereas an electron micrograph is a photograph, or a print, so that what is dense, or black in your electron micrographs, is white in the microradiograph. I just want to clarify this point.

TALMAGE: Dr. Robinson, these figures show primarily osteoblasts bordering the canal. If these osteoblasts are actively synthesizing collagen, what prevents the center from filling in with matrix? How does it maintain its constant size?

ROBINSON: As an osteon is formed, a state is reached in which no more closure or encroachment on the vascular space in the center of the osteon occurs. Equilibrium is established.

TALMAGE: Are not the osteoblasts still synthesizing collagen?

ROBINSON: Figure 13 happens to be from a puppy. It has obviously reached a point where, if the peripheral osteoblasts synthesize much more bone matrix, they would shut off their own fuel supply. I think that some factor comes into play at this point which prevents this. I do not know what the factor is.

CURREY: Are three distinct layers usually found in a fully mature haversian system?

ROBINSON: Even about the periphery of a fully mature section of a haversian canal one usually can find three distinguishable zones along some segments of its periphery (fig. 127). In this regard, great care must be taken during the preparation of the material to avoid any demineralization. When these sections of bone are produced by a special microtome, the section is only about 500 angstroms thick. These small sections float out as a ribbon over a water bath, and then

they are picked up on a grid. If the pH falls below about 6.8, these sections will demineralize very rapidly. In this work it is essential, therefore, to keep the pH elevated.

Boothroyd (ref. 26) wrote a paper about this subject, and Dudley and Spiro (ref. 27) earlier pointed out the problem of demineralization on the surfaces of these matrix masses in such thin sections. Even with great care, one must always be suspicious that some demineralization could have occurred. However, I think we have this problem under control and do not feel that this area of unmineralized matrix is an artifact.

ARNAUD: How do you control the problem?

ROBINSON: We place an indicator in the fluid and we keep the pH at 7.2.

ARNAUD: What happens if it changes?

ROBINSON: We change our solution. As the color starts to change we change our solution.

BÉLANGER: Is there a possible loss at pH 7.2?

ROBINSON: We have not seen any, whereas the loss becomes very dramatic below pH 6.8.

OWEN: In figure 13 it seems as if there is a distinct boundary between the three zones, rather than a gradual merging of the zones. Is this correct?

SAXÉN: Would you indicate the cell surface, or the cell membrane?

ROBINSON: Figure 14 is a more highly magnified electron micrograph than figure 13, and shows more clearly the plasma membrane of the osteoblast in relation to the bone matrix. This figure shows the plasma membrane covering the protoplasmic extensions of the cell which proceed into the bone matrix.

The crystal masses start to form spottily through the mineralized zone. Nevertheless, there is a clear demarcation from the next zone, in which mineralization is fairly good, but definitely incomplete in some areas; then, in the next zone mineralization is suddenly quite complete.

BUDY: In figure 14, what are the vacuoles below, above, or in between the mineralized area?

ROBINSON: Those are collagen fibrils, and the space between the fibrils is more dense in this micrograph than the fibrils themselves. This may be because the embedding media picked up some of the lead counterstain, whereas the fibrils themselves did not pick up so much.

If the mineralization were all peripheral to the collagen fibrils, one would see holes the size of the collagen fibrils throughout the mineralized matrix mass when the mineralized matrix mass is sectioned transverse to the long axis of the collagen fibrils. We have not seen

these holes, and this is one of the major arguments for the belief that these mineral crystals occupy space in the fibrils, as well as around the fibrils in the mineralizing region.

PECK: You cannot exclude the possibility, though, that those spaces are occupied by lipid.

ROBINSON: The collagen fibrils are laid down around the osteoblasts that line the haversian canal. These osteoblasts are laying down the fibrils in one direction, but apparently every few hours they switch the collagen axis and lay them down more or less at right angles to the previous direction. The fibrils are usually more osmiophilic than the material in the regions between them. Therefore, I have no evidence that there is much lipid present.

MACDONALD: Dr. Robinson, would you say this is evidence that calcification not only occurs at the surface of the mineralization front, but also can occur at random in the matrix above it?

ROBINSON: That is correct. The mineralization front is not observed to be a straight line that moves from the fully mineralized matrix into the newly formed matrix. Rather, mineralization first appears spottily throughout the newly formed matrix. The cell produces the matrix; then there is a lag period. This lag period can be extended in several situations. One of the most clinically outstanding situations is the fracture callus, where a great deal of matrix mass may be formed before mineralization appears.

Cameron has mentioned this (ref. 28), particularly in the fracture callus of the rat.

NICHOLS: How does the mineral get there, by diffusion?

ROBINSON: Right. First, the matrix mass does not decrease or increase in volume during mineralization, and second, the density increases rather suddenly in those particular regions where mineralization is occurring. This increase implies diffusion of mineral ions into crystal nucleation sites at the time of mineralization and not significantly before. On the basis of electron micrographs of mineralizing matrices of epiphyseal cartilage and of bone, as previously noted (refs. 21 and 29), an amorphous "cloud" more dense than the surrounding ground substance seemed to appear about a portion of one collagen fibril or about adjacent portions of several fibrils. It was in this cloud and in relation to a collagen fibril that the first crystals seemed to appear. The most logical explanation is that a chemical change occurs in a portion of the newly formed bone matrix. This change involves the collagen and a surrounding portion of ground substance. The altered portion of the bone matrix then becomes a "trap" for calcium and phosphate ions. Diffusion into this trap is possible because the water bridge for calcium ions is open between the matrix trap and the vascular space.



In 1963 I speculated that an enzyme produced by a cell might, as suggested by Campo (ref. 23), split the noncollagenous protein from the mucopolysaccharide, permitting chondroitin sulfate to act as a calcium trap while the adjacent collagen fibril became a phosphate trap (ref. 30).

If the calcium ions were already in the matrix and if only the water was removed at the time of mineralization, a vacuum would be formed or the matrix would decrease in volume. Furthermore, if calcium ions were already in newly formed bone matrix at a density even approaching the density they assume during apatite crystal formation, microradiographs of freeze-dried bone and microchemical analyses should show their presence, but they do not (ref. 24).

It appears to me that the mineralization process is instituted in bone matrix subsequent to bone matrix formation and by a quite separate mechanism. During this process the mineral ions mainly replace the matrix water, volume for volume, as they concentrate in the organic matrix focally and form hydroxyapatite crystals. These ions must diffuse into the matrix during the mineralization process and not significantly before.

This organic matrix mass produced by the osteoblasts has a high water content. The extracellular, extravascular fluid is conceived to form a water bridge from the vascular space right into this newly formed bone matrix. Thus, rapid diffusion of ions, such as those of calcium, is possible prior to matrix mineralization and during matrix mineralization. This diffusion slows as full mineralization is approached.

NICHOLS: I would like to start a discussion on this point. Your theory would seem correct, except that it must be remembered that the matrix is laid down by cells in a fluid atmosphere which contains ions; suddenly, something happens so that these ions aggregate into crystals. It seems to me that at this time one of several things may have happened. It could be that it starts very full of water, as your studies show, and something pumps out the water, leaving the silt to accumulate to the point where the mineral precipitates. A second possibility is that something is happening to that matrix which is modifying it *in situ*, or maturing it. There might be a crosslinking or some extracellular enzymatic processes going on (refs. 31 and 32). The third possibility is that the cells located at the site "get the word" and start pumping ions into the area, instead of their pumping water out of the area. It seems to me that one should believe in one of these three possibilities.

BÉLANGER: There may be a possibility also that a local change in the pH would be attributable not to the collagen but to the rest of the organic matrix, particularly the mucopolysaccharides. This is known

by the histochemical approach. The mucopolysaccharides that are close to the surface are mostly of the acidic variety, while those more centrally located seem to respond better to methods such as the periodic acid-Schiff, which indicates a neutral variety.

NICHOLS: Could you define what you mean by central and peripheral?

BÉLANGER: Yes. What is away from the osteoblast I call more central, and what is closer to the cell I call peripheral. In this bone, which Dr. McLean calls "prebone," are found a number of these acidic-staining mucopolysaccharides. This is like the situation in young cartilage; calcification cannot occur in this area because of the environment and the low pH. But as one moves away from this area and the cells become older, the tissue can make no more of them. When the cell is older and more mature, it acquires equipment to make other types of mucopolysaccharides; consequently, I think Dr. Robinson is right to think that collagen is far from being the only factor involved in the secretion of the cells away from the surface.

NICHOLS: When you say "more acidic," do you mean there is more bound sulfate at the cell surface?

BÉLANGER: This is one type. This is one way of defining these typical mucopolysaccharides. Another way of looking at them is that they would have other acidic groups apart from sulfates.

NICHOLS: Then you have to explain why the mucopolysaccharides become neutral.

BÉLANGER: I cannot explain that. I do not say that they do become neutral. But if this environment is constantly changing, it is changing under the influence of the cells which are located in one part or another of the bone. The cells at the surface possibly can make more mucopolysaccharides with sulfate than the cells which are deeper inside; the osteocytes which, because they are more mature, can now make more of another type which is more neutral, and will change from one area of bone to another.

NICHOLS: I think Dr. Howell has some data which bear on this subject.

HOWELL: Dr. Nichols was referring to our pH measurements on an essentially extracellular fluid phase sampled from normal and rachitic rats *in vivo* by a micropuncture technique to be described later. The fluid samples, about 20  $\mu$ l, have been shown to originate from the hypertrophic cell cartilage; that is, predominantly, we believe, from or close to histologic sites of calcification. With precautions to prevent loss of CO<sub>2</sub> the pH is measured directly with microelectrodes on such fluid sampled before and after various measures to promote calcification. Prior to healing, pH recordings in the fluid have been 7.45 to 7.55; during healing these readings increased to 7.50 to 7.58, the same as for fluid aspirated from normal cartilage plates

or from perichondrial "lymph" on rachitic cartilage plates. We think that our model of endochondral calcification may have an important bearing on mineral transfers in bone matrix.

HOLTZER: Is not this point leading up to something; namely, that the cell knows what is top and bottom and what is right and left? The polarization of the osteocytes is exceedingly relevant. It is clear that the cell does not want to make collagen in the area facing the crystallization zone. In short, the whole system is spatially oriented. The cell is commanding and determining where and when things are to occur both inside and, probably indirectly, outside the cell. To visualize this as simple precipitation in an ionic cloud would be denied by that very photograph (fig. 14), although later you might find that the cell decided that what is left field will not be right field. In short, when the cell is completely surrounded by matrix, something must happen to that cell and it says, "Now instead of sending collagen off to the right I am going to send it, or its precursor, off to the left."

NICHOLS: I think your point is very well taken, but there is another factor that I would like Dr. Bélanger to comment upon. I also think that there is a change in pH, and I should have included it. I wonder what role the glycolytic metabolism of these cells may play in this whole process. One can explain the failure of mineral precipitation of nucleation by simply invoking a lower pH around the cells without involving sulfation at all. All that is needed is a cell that happens to excrete a lot of organic acid, which we now know these cells do.

ROBINSON: I would like to complete the rest of the picture of this matrix mass surface and point out the roughness factor of the periphery of the matrix mass available to extracellular fluid on the walls of lacunae and canaliculi.

Figure 15 shows an osteocyte in a lacuna in a quite completely mineralized mass of bone matrix. This osteocyte is not very close to a periosteal, endosteal, or vascular canal surface; it is quite deeply buried. Osteocytes in such a position show atrophy of their cytoplasmic organelles; e.g., endoplasmic reticulum membrane system and mitochondria. The cells occupy less of the lacunar space. The matrix bordering the lacuna is apt to be more completely mineralized, although along one edge of this lacuna some unmineralized collagen fibrils are present. The extracellular fluid space between the cell and the bone matrix wall of the lacuna is greater than that about a newly buried surface osteocyte. (See fig. 12.) Note that at the electron-microscope level of tissue organization, the crystals produce a fairly rough and irregular surface along the periphery of the lacuna.

The protoplasmic-cell extensions can be seen proceeding out from the cell body toward the bone matrix, and one of these in figure 15 can be observed particularly well. The protoplasmic extensions do not

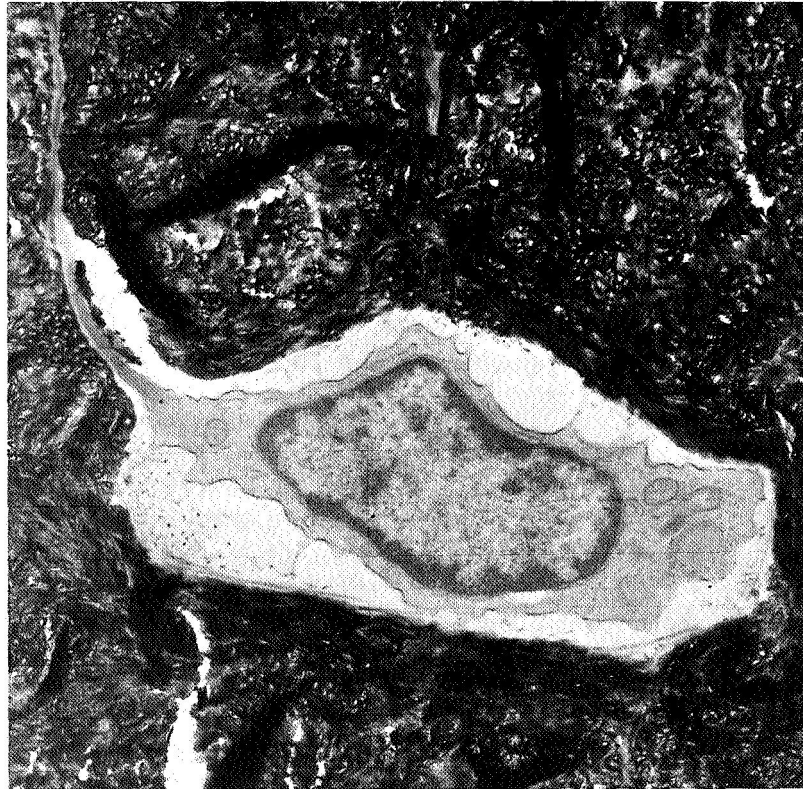


FIGURE 15. Electron micrograph of a deeply buried osteocyte in the periphery of a well-mineralized osteon in the midcortex of the diaphysis of the femur of an adult dog. These osteocytes, unlike the surface osteocytes illustrated in figure 12, show atrophy of the rough endoplasmic reticular membrane system and a marked reduction in number of mitochondria. Unmineralized collagenous fibrils are seen along the periphery of this lacuna, although unmineralized matrix is near minimum in this particular situation. The cell extension can be seen passing up to the top of the figure on the left. Approximately 6780  $\times$ .

completely fill the canals in which they lie; there is always a small extracellular space between the plasma membrane that surrounds each one and the mineralized matrix wall of the canal. Baud (ref. 33) also found this space between mineralized matrix and the tube of cell membrane in canaliculi.

CURREY: This is not a fixation artifact? Can you be sure of that?

ROBINSON: I do not think this is an artifact. Lipp (ref. 34) in extensive light-microscope histochemical studies, demonstrated a sleeve of material in this region of canaliculi between cell membrane and bone matrix which had a high mucopolysaccharide content.

Figure 16 is a cross-sectional view of several canaliculi in quite fully mineralized bone matrix. It is interesting that one often sees two protoplasmic extensions in one canal in canine bone. We have searched for the junctions between the protoplasmic processes of osteocytes, and osteocytes and osteoblasts in canaliculi, and we have observed several of these (ref. 25). We have seen no specialized areas, such as desmosomes, on abutting plasma membranes of the two bone cells, but we have seen such specialized regions of cell membrane at the contact areas of endothelial cells. (See fig. 128.)



FIGURE 16. Fully mineralized bone matrix from the cortex of dog femoral diaphysis. The canaliculi are obvious. Many contain two cell extensions. The extracellular fluid space about these extensions is the clear white space between them and the black mineralized matrix. The collagen periodicity is emphasized in this very thin section by tiny bone crystals in the fibrils. Fascicules of larger and more compactly arranged crystals can also be seen paralleling the fiber axis; they are thought to be between the collagen fibrils. Approximately 12 000  $\times$ .

Note the roughness of the surface of the canalicular wall produced by the little crystals in the periphery of the mineralized bone matrix. On the other hand, one should note that the mineralization of the bone matrix extends right up to the edge of the bone matrix on these canal walls, and unmineralized collagen fibrils are not observed along the walls of these canals in normal dog bone. However, along the walls of haversian canals and lacunae some unmineralized bone matrix is usually seen.

In contrast to this region of quite full mineralization of bone matrix in the middle of the cortex of a dog femoral diaphysis, I should like to return to Dr. Cameron's electron micrographs (figs. 11 and 12), where one can observe newly formed bone matrix very well. They happen to be from the surface of a metaphyseal trabecula of a growing rat, but they are good examples of partly mineralized bone matrix. In the areas of very incomplete bone matrix mineralization between foci of mineralization, many of the crystal surfaces are available. However, when mineralization is quite complete, we believe that the surfaces are no longer available because they have been shut off from rapid diffusion of calcium ions by, for example, the very fact that the water bridges necessary for the transit of these ions have been obliterated. However, in these incompletely mineralized areas, the water bridge is still open between vessel and crystal surface.

It is in these regions that tetracycline molecules and calcium ions are known to be deposited. We feel that the mechanism is quite simple; namely, that as long as a water bridge extends to the crystal surface, then the ion or molecule that has some chemotactic attraction for the crystal surface can reach the crystal surface until mineralization approaches completion. In other words, even just the surface of the mineralized matrix is a hotspot, but it is only a hotspot in a shallow sense. When very incomplete mineralization extends deeply enough through a bone matrix region, then you have water bridges in depth to all of the crystal surfaces, and you have what is known as a hotspot in autoradiographs as viewed in the light microscope.

HOLTZER: In figure 12, must you not postulate that there is actually an inhibitor here? I am amazed to see how close the crystalline area approximates the cell membrane of the cell top. There is a collagen layer, presumably, on the cell bottom.

ROBINSON: This cell used to be an osteoblast, and it is being hemmed in. This cell is creating a new matrix in this region.

HOLTZER: Yes. There must be a zone that actually cannot calcify.

URIST: What is the composition of the osteoid seam?

ROBINSON: It is collagen, water, and mucopolysaccharides.

URIST: Is this area filled with crystalline calcium phosphate? Is

it possible that this space could have been filled with noncrystalline calcium phosphate?

ROBINSON: I would like to postpone an answer to that; however, I will tell you what I feel.

We held up one paper for publication for a long time to test this point. After injecting calcium tracer into the heart of a rat and sacrificing the rat, we took slices of the tibia. We sacrificed at two time intervals, one at 2 minutes and another at 2 hours. The tracer calcium, we would think if it were going to such a pool of ions, would be quite readily soluble in the fixatives or in a fluid which is used in a dehydration bath.

URIST: Was the tissue fixed in osmic acid?

ROBINSON: No; in glutaraldehyde.

URIST: What was the pH of the solution?

ROBINSON: We never use a fixative with a pH below 7.2 whether we use glutaraldehyde or osmic acid. Both are buffered. Incidentally, if phosphate buffers are used some very peculiar pictures are obtained, so you must still be careful not to use phosphate buffers.

URIST: What kind of peculiar pictures?

ROBINSON: We start to see a great deal of mineralization in this area of unmineralized collagen.

URIST: The question is, Is there a physiologic osteoid? Does this physiologic osteoid represent collagenous matrix that contained noncrystalline calcium phosphate? The significant statement that you have made about the pH of your fixative would suggest that if such a thing does exist it would be soluble at pH 7.2. Are there calcium phosphates that are soluble at pH 7.2?

Electron micrographs do not answer Dr. Bauer's question about whether mineral *in vivo* is or is not crystalline. I think you have shown, and I agree, that bone mineral is crystalline, but I wonder if a non-crystalline material might occupy the osteoid seam. In addition to such factors as pH of the solution, is it necessary to take into consideration such factors as leaching of loosely bound deposits of mineral?

ROBINSON: If this substance exists in any concentration, one would expect that it would show up as greater density in this area. Therefore, we did this experiment that I just described. It is true that when you take sections of the tibia at 2 minutes, collect all the fixative dehydration baths, at the end of that dehydration take the remaining sample and demineralize it, put the solutions onto planchets, and count the total radioactivity of the solution, you find that in the 2-minute specimen you lose about 16 percent of the total radioactive calcium. At 2 hours, it is less than 1 percent.

In view of this experiment—and admitting that this is only one

experiment—I do not feel that the preparation method washes out a significant amount of calcium from that space.

URIST: Have you done an experiment in which you have measured the calcium content of the fixative?

ROBINSON: Yes; and also measured that of the total specimen.

URIST: So that you would account for all the calcium that was there, and you would account for the calcium that could have come off in the fixative; and thereby, you feel that you have excluded the possibility of noncrystalline calcium phosphate in the tissues?

ROBINSON: No. I do not feel that we are losing any significant quantity of calcium, because at 2 minutes after injection into the bloodstream there is still a good deal of the calcium in the extracellular fluid and in the vascular and marrow spaces; this calcium has not yet reached a crystal nucleation locus or the surface of a crystal in the bone. The reason we performed this experiment was that we had noted that as early as 2 minutes after the injection of the isotope, it was largely associated on autoradiographs with the crystalline material that we see on the periphery of the mineralized bone matrix masses, and not in that area between the cells and the crystals. I cannot say that we do not have a load of calcium, perhaps, attached to a mucopolysaccharide in this area between the bone cell and the bone crystals in the mineralized bone matrix; but I think this experiment counteracts to some extent the arguments that we are washing out a large quantity of calcium and that we are not getting a good representation of what really occurs in combined autoradiographs and electron micrographs in regions of mineralizing bone matrix.

URIST: I do not think we are inquiring about a large amount of calcium phosphate. I am inquiring about whether there is a critical transitional phase that is leached out of the tissue.

COPP: I think that you are both right. There could be calcium and phosphate in the space between the cell and the crystals which could be washed out, but we do not know how much.

ROBINSON: Obviously, calcium ions have to pass through the space from the vessel to crystal nucleation sites in bone matrix, but we doubt that calcium is concentrated in large amounts in the bone cells or throughout the unmineralized bone matrix prior to its demand at crystal nucleation sites in specific regions of the mineralizing bone matrix.

PRITCHARD: Some years ago Horning (personal communication), using microincineration, found a surprising amount of calcium in osteoid. I remember thinking there must have been a mistake; but it was true.

COPP: Obviously this is a transitional stage.

URIST: This question is a very important part of the subject of



calcium homeostasis. A recent paper by Kashiwa (ref. 35) purports to show that with the dye, glyoxal bis(2-hydroxyanil), known as GBHA, it is possible to demonstrate calcium in noncrystalline form in the proteinaceous material in the cytoplasm of osteoblasts and osteocytes. If this is true, it means a whole new area containing a small, but critical, fraction of the total body calcium is present in bone cells. The amount present in osteoid seams is not revealed because the stain does not fix calcium phosphate complexes or apatite.

Dr. H. K. Kashiwa was invited to the Bone Research Laboratory at UCLA to give instructions on his method, and we learned that he was using high concentrations of GBHA to stain whole mounts of the bone of the rat calvaria. I made thin sections of undecalcified bone and preparations of tendon with and without tendon-calcium ion complexes; I stained them by Kashiwa's technique, except with only one-tenth of the concentration that he employed, and obtained the photomicrographs shown in figures 17(a) and (b), and 18(a) and (b).

The dark-red staining, insoluble calcium-GBHA complex could be observed in the cytoplasm of osteoblasts and osteocytes; the osteoid seams and the calcified intercellular matrix were unstained (fig. 17).

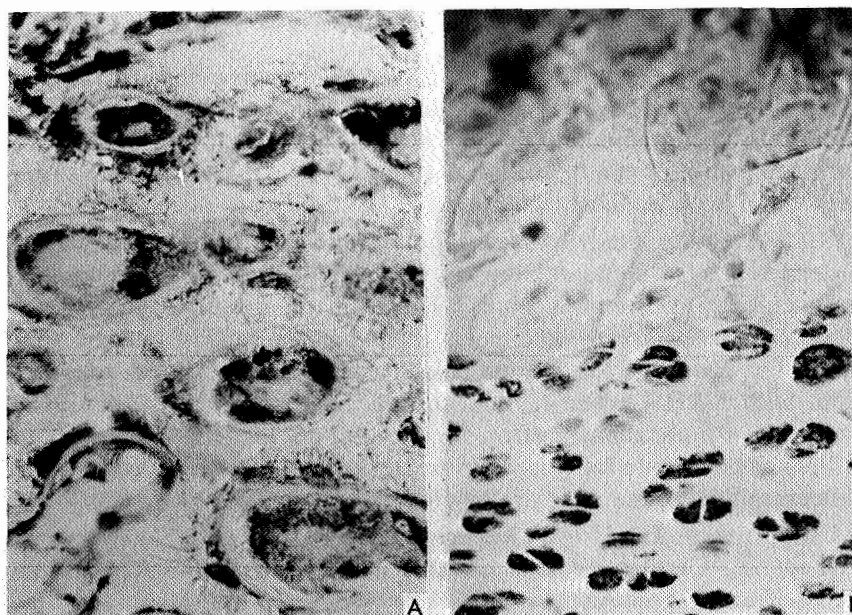


FIGURE 17. Photomicrographs of undecalcified sections of (a) tibial shaft cortex and (b) epiphyseal cartilage from a newborn rabbit. The sections were stained with a dilute solution of GBHA.

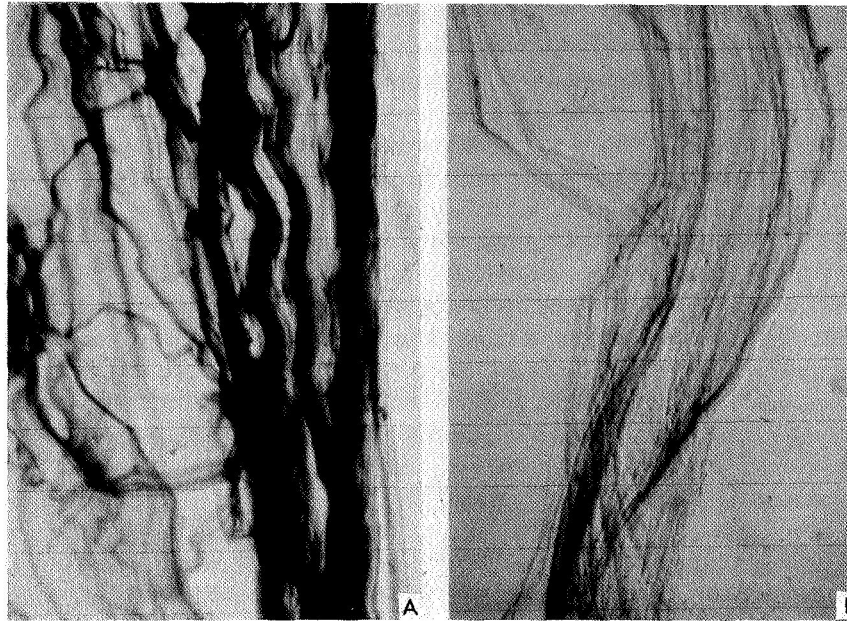


FIGURE 18. Photomicrographs of rat-tail tendon treated with (a)  $\text{CaCl}_2$  and (b)  $\text{CaCl}_2$  and phosphate buffer. The sections were then stained with GBHA.

The GBHA also stained the cytoplasm of chondrocytes (fig. 17(b)). The chondrocytes may be secreting a fibrous protein-calcium complex similar to that shown in figure 18(a). The calcium in uncalcified cartilage matrix or apatite in cartilage matrix is not available for reaction with GBHA under these conditions.

Rat tail tendon, treated with 5 millimoles per liter of  $\text{CaCl}_2$ , is stained a deep red by a dilute solution of GBHA (fig. 18(a)). The tendon-collagen-calcium complex binds GBHA in an insoluble precipitate. Tendon untreated with calcium is unstained or weakly stained with GBHA. The tripartite tendon-calcium-phosphate complex does not stain with GBHA when tendon collagen is treated first with  $\text{CaCl}_2$  and then with phosphate buffer, 5 millimoles per liter (fig. 18(b)). The affinity of phosphate for calcium is greater than that of GBHA when low concentrations of the stain are employed.

COPP: I think Dr. Howell has some data on this point.

HOWELL: Cartilage data show this increase in calcification up to the ossification process.

COPP: Dr. Robinson's magnificent electron micrographs of bone have given us some idea of the problems and interactions between bone mineral and extracellular fluid. We have found, for example,

that the plasma calcium dropped 30 percent after thyroparathyroidectomy in the rat, and there was a corresponding drop in exchangeable bone calcium (ref. 36).

BAUER: That is your computation from the accretion rate?

COPP: The accretion rate also changed.

BAUER: I wonder if there is any experimental evidence that alterations in exchangeable bone calcium do occur. You say that the accretion-resorption rate did not change. However, I think it is right to say that no one has seen a change in the exchangeable amounts without some change in the accretion-resorption, so it is more of a concept than actually based on evidence.

COPP: I do not agree. When calcium is injected into a thyroparathyroidectomized dog, as in figure 19, a new equilibrium is reached at a higher plasma calcium level. There is a corresponding increase in the labile bone calcium pool, which we estimate to be 50 to 80 mg/kg.

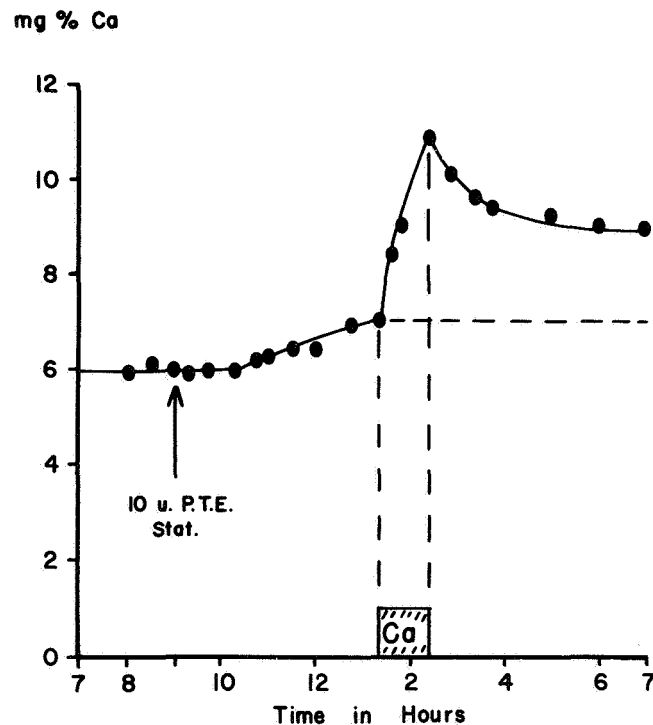


FIGURE 19. Increase in the equilibrium level for plasma calcium after intravenous infusion of calcium (10 mg/kg) into a dog thyroparathyroidectomized 1 week earlier. The dog also received a dose of parathyroid extract sufficient to raise the plasma level 1 mg/100 ml, as indicated by the broken line.

BAUER: I had estimated it to be 5 grams per 100 units.

COPP: No. We estimate the pool on the basis of body weight, and it is interesting to note that our estimate agrees quite well with your estimates of exchangeable calcium as measured with radioactive isotopes.

CURREY: I would like to ask a question about this labile pool which I do not quite understand. It seems to me that the labile pool is a surface phenomenon; therefore, why do you say that the amount in the labile pool is a measure of change?

COPP: Your point is well taken. The pool really represents a volume capacity; assuming arbitrarily that the concentration of calcium in this labile bone storage pool is the same as in plasma, we would estimate a pool size of 500 to 800 mg/kg body weight. At a normal plasma calcium level of 10 mg/100 ml, the amount of calcium in this pool would be 50 to 80 mg/kg. Assuming the capacity of this pool is unchanged, an increase of 2 mg/100 ml in plasma calcium would correspond to an increase to 16 mg/kg in the calcium in this pool.

There are two distinct and different points of view with respect to the exchange between the calcium in bone and the calcium in extracellular fluid. We may not be able to resolve these differences in the time available, but they should be clearly stated.

Our point of view is that those crystals of bone salt which are accessible to the circulation act like an ion exchange column, with calcium on the surface of the crystals exchanging with the calcium in the surrounding fluid by a purely physicochemical process. This would correspond with the almost universal uptake of  $^{45}\text{Ca}$  around all the vascular channels of bone observed by Dr. Rowland a few minutes after injection of the isotope. As with any ion-exchange column, if the ionic concentration of calcium in the fluid increases, the concentration of calcium on the crystal surface will increase. This, we feel, corresponds to our labile bone storage pool for calcium. The other view holds that there is a regulating biologic membrane separating all bone mineral from the surrounding fluid; the level of calcium in the fluid depends on this membrane and the effect that the calcium active hormones have on it. In both cases, it is agreed that there are biologically active and hormone modified processes of accretion and resorption which determine the ultimate calcium balance between bone and body fluids.

ARNAUD: I do not think that we have enough information or the techniques to distinguish adequately between these processes.

COPP: Dr. Talmage would like me to mention the parathyroids, which he thinks are related to calcium homeostasis. Their importance was indicated by the classic work of Hastings and Huggins (ref. 37). The brilliant hypothesis of McLean (ref. 38), and the experiments

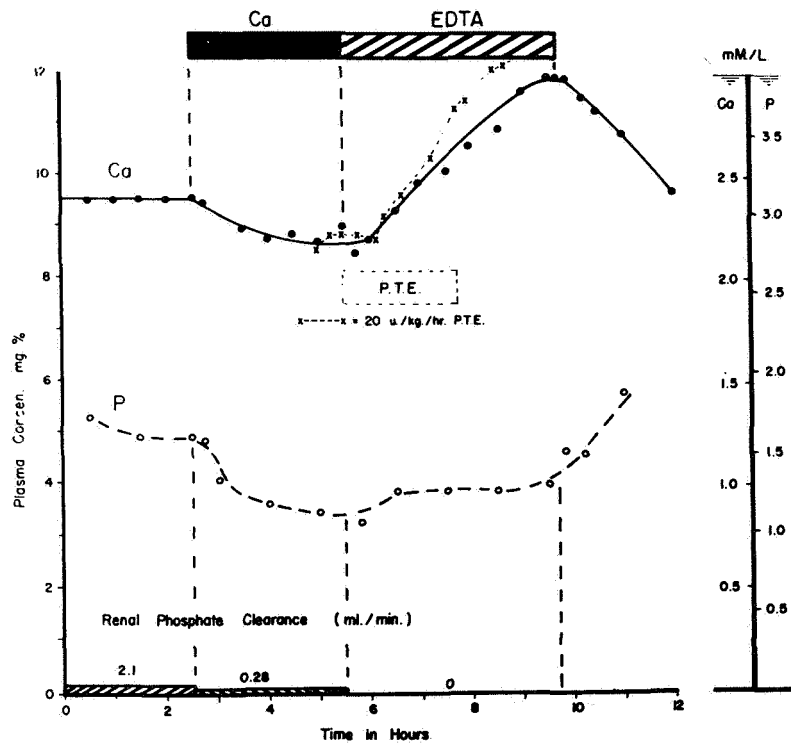


FIGURE 20. Typical response of systemic plasma calcium and phosphate levels to perfusion of parathyroid with blood to which calcium or EDTA has been added. Dotted line shows changes in the same dog when parathyroid extract (PTE) (20 units/kg/hr) was injected for a 3-hour period 6 hours after thyroparathyroidectomy. [From ref. 39; reprinted by permission of the publisher.]

of Copp and Davidson (ref. 39), clearly showed that this was accomplished by negative feedback control. When the thyroid and parathyroid glands of a dog (fig. 20) were perfused with blood low in calcium, there was a rise in systemic plasma calcium which closely resembled the response obtained in the same animal with continuous intravenous infusion of 20 units/kg/hour of parathyroid extract. This is approximately 200 times the maintenance output of the parathyroid hormone as determined by us in previous experiments (ref. 13). These values are generally in line with the observations of Sherwood et al. (ref. 40) and Care et al. (ref. 41) on parathyroid hormone production in cows, sheep, and goats in which the glands were stimulated or inhibited by injection of EDTA or calcium. The hormone in plasma was determined by radioimmunoassay.

In our studies on perfusion of the isolated glands and in studies

on increased mobilization of calcium during calcium infusion, as shown in figure 21 (ref. 42), it is apparent that a fall of as little as 10 to 15 percent in the level of calcium in the blood passing through the gland is sufficient to stimulate hormone production.

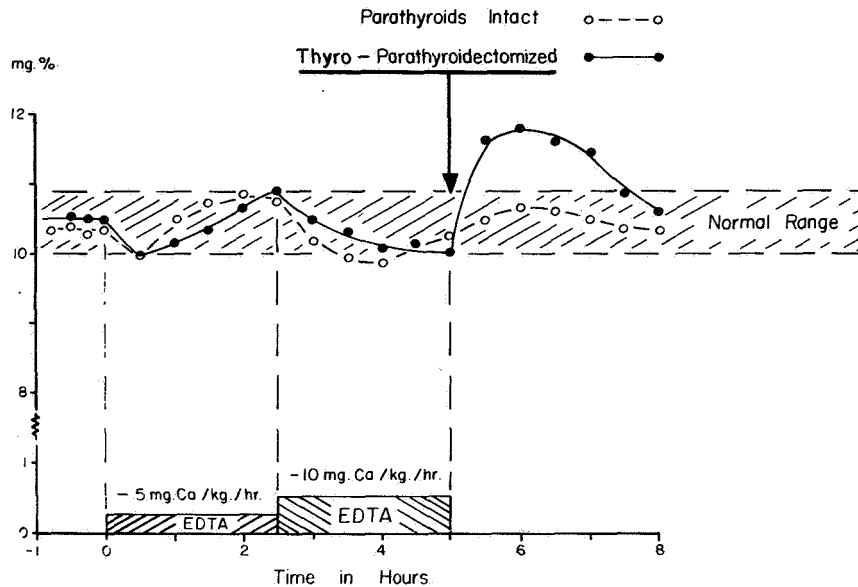


FIGURE 21. Effect of EDTA infusion on plasma calcium level in the same animal with intact glands (broken line) and after removal of the thyroid and parathyroids immediately after EDTA infusion as indicated by the arrow. [Adapted from ref. 42; reprinted by permission of the publisher.]

Dr. Raisz has done some beautiful studies on the effect of the calcium concentration on parathyroid cells in organ cultures (ref. 43). Dr. Raisz, would you comment on this work?

RAISZ: I think we are now at the point where an elegant servo-analysis of calcium homeostasis could be achieved if we could get precise-enough data to satisfy mathematicians. One of the difficulties in a biologist's work is that he can never satisfy the mathematical demands for large numbers of points, but he can make an attempt. I think a model, on the basis of which we can make a specific analysis, is available. I would like to make the simplifying assumption—which we can debate—that the controlling system in calcium regulation is bone resorption and not bone formation. Bone formation is likely to be controlled by matrix synthesis, by hormones which regulate bone growth, and by local factors which determine the structures of bone; one might call these the orthopedic functions of bone.

NICHOLS: When you speak of "bone resorption," do you mean both mineral and matrix or just mineral?

RAISZ: I will talk about mineral, but it is obvious that one cannot remove adequate amounts of mineral from bone without getting rid of some of the matrix.

URIST: Are you referring to second-to-second control of calcium homeostasis?

RAISZ: No.

URIST: Is matrix formation involved in second-to-second control?

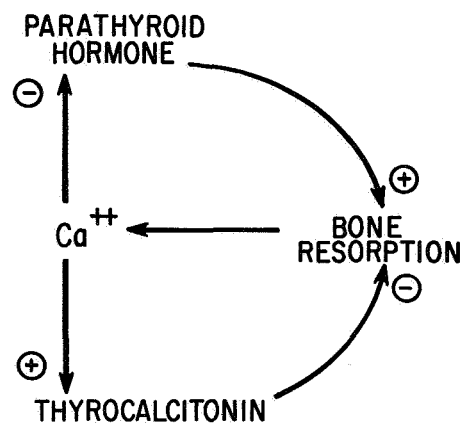


FIGURE 22. Illustration of the simplest model for feedback regulation of blood calcium ion concentration in which bone resorption is the major controlling system. In addition to the feedback control of parathyroid hormone, the possible role of thyrocalcitonin is indicated.

RAISZ: No; it is not.

URIST: I would think certainly hour-to-hour control required matrix formation, and that you are correct in assuming that second-to-second control is a function of the mineralized surfaces.

RAISZ: Given bone resorption as a controlling system and calcium ion as the controlled system, we have, in the McLean hypothesis, negative feedback control through parathyroid hormone which is constantly secreted and stimulates bone resorption. These are all things we have known since the 1920's. (See fig. 22.) Now we can ask what kind of control this is. We have only fragmentary evidence at present, but as investigators get better at studying the way in which the parathyroid gland responds to calcium, they can ask questions of the

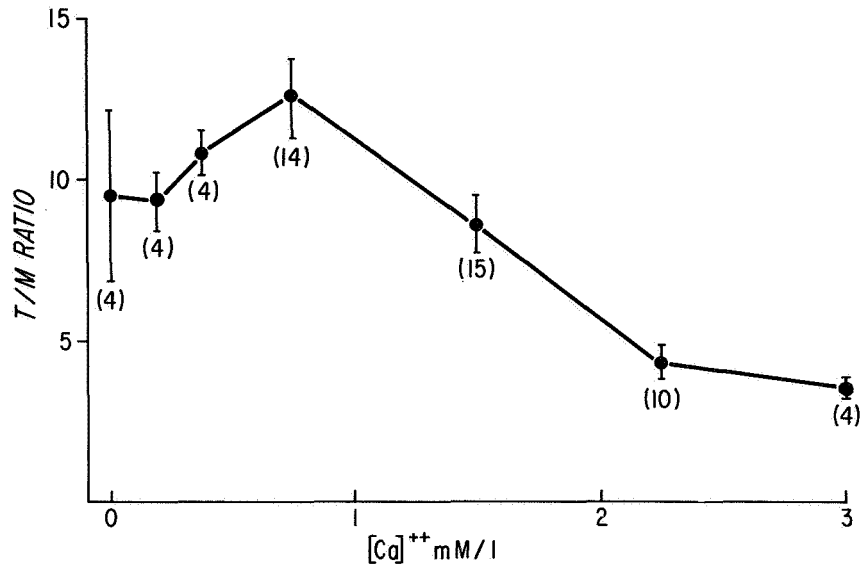


FIGURE 23. Effect of calcium concentration on tissue/medium concentration ratio of  $^{14}\text{C}$ -alpha-aminoisobutyric acid after 2-hour incubation. Points are mean values with SE. Number of observations is given in parentheses. [From ref. 44; reprinted by permission of the publisher.]

kind which have been asked about such feedback mechanisms as the carotid sinus control of blood pressure. For example, is there an amplified proportional control whereby small changes in calcium level cause proportionately large changes in parathyroid hormone output? I think we can assume that parathyroid hormone has two ways of functioning. One is an amplified proportional feedback which responds to minute-to-minute changes in serum calcium concentration, both by changes in secretion and changes in hormone synthesis. The second is an integral response which requires growth of new cells or possibly involves activation of cells which have been in a quiescent condition. These are probably general phenomena of endocrinology. This is illustrated by the feedback control of the uptake of alpha-aminoisobutyric acid (AIB), a nonmetabolized amino acid analog which is actively transported into the cells by the system responsible for the uptake of the natural amino acid, glycine (ref. 44) (fig. 23). These data were obtained from isolated rat parathyroid glands incubated for 2 hours at varying calcium concentrations. Normal calcium concentration would be about 1.5 mM. Between  $\frac{1}{2}$  and  $1\frac{1}{2}$  times this concentration, there is a steep curve relating AIB uptake inversely to calcium concentration. With lower or higher concentrations beyond this range,



there is little further change in AIB uptake. These data, then, follow an S-shaped curve which (while not as clear cut as the data for the carotid sinus regulation of blood pressure) does indicate a proportional response of the parathyroid to calcium concentration which is amplified around the normal calcium concentration of blood. To prove this, extensive data on the effect of calcium on the rate of secretion *in vivo* will be required. Assuming that such a response exists, maximal stimulation occurs at half normal calcium concentrations; the only way in which the parathyroid can respond further to prolonged stimulation would be to increase its functional tissue mass. Figures 24 to 26 illustrate such a response. The model is the vitamin-D-deficient rat in which, because of end organ failure, i.e., the inability of bone tissue to respond to parathyroid hormone, one can see prolonged hypocalcemia over many days (ref. 45). With hypocalcemia there is an

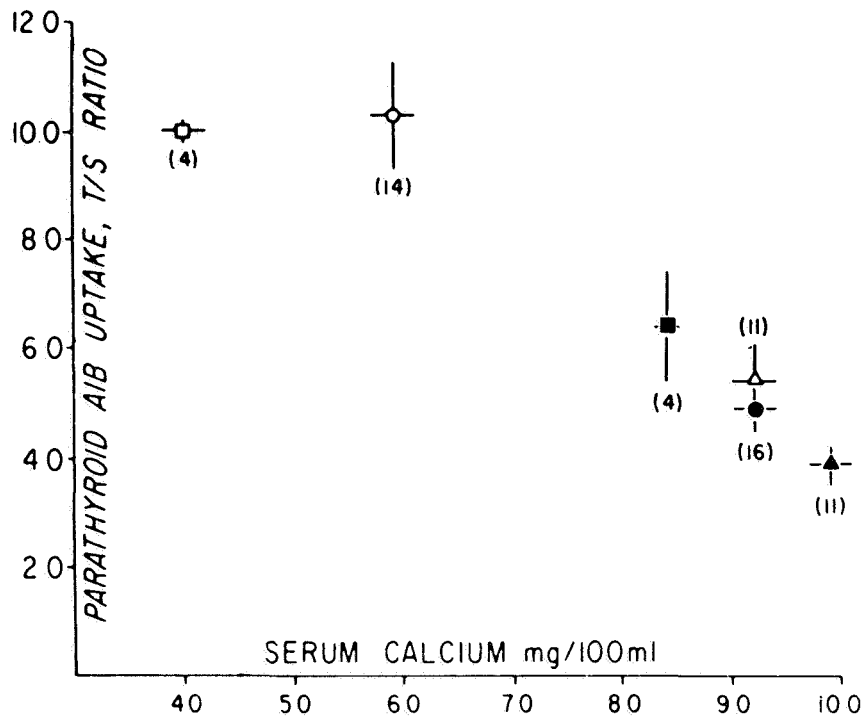


FIGURE 24. Relationship between serum calcium concentration and parathyroid uptake of AIB expressed as the ratio of AIB concentration in parathyroid tissue water to that in serum (T/S ratio) at equilibrium 24 hours after AIB injection. Each symbol represents mean and SE for both serum calcium concentration and parathyroid AIB uptake. Figures in parentheses represent number of animals in each group. Symbols for different diets are indicated in figure 25. [From ref. 45; reprinted by permission of the publisher.]

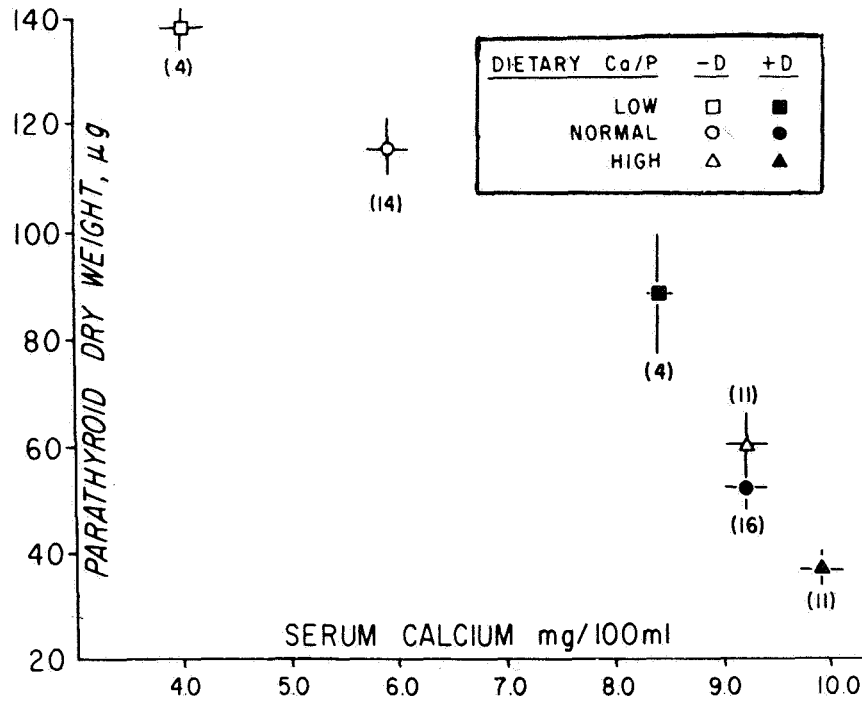


FIGURE 25. Relationship between serum calcium concentration and parathyroid dry weight. Legend indicates whether animals were on vitamin D-deficient (-D) diets or on the same diet with vitamin D supplement (+D), 80 I.U. every 3 days. The diets were as follows: low Ca/P=0.03% Ca and 0.1% P; normal Ca/P=0.4% Ca and 0.3% P; and high Ca/P=0.8% Ca and 0.1% P. [From ref. 45; reprinted by permission of the publisher.]

increase in parathyroid AIB uptake as the serum calcium falls to 6 mg/100 ml (fig. 24). With further lowering of serum calcium, achieved by combining vitamin D deficiency with a low calcium intake, the serum calcium falls still further, but there is no further increase in the function of the tissue as indicated by the AIB uptake. However, the gland can respond by getting bigger. Figure 25 illustrates the inverse relationship between parathyroid size and serum calcium for both dietary changes in calcium and for the effects of vitamin D deficiency. At the risk of overanalyzing our data, we have taken the activity of the gland, as measured by amino acid uptake, and the size of the gland, multiplied them together, and divided by body weight to obtain a parathyroid activity index. Figure 26 illustrates the remarkably good inverse linear correlation between calcium concentration and the parathyroid activity index. This linear response is a reflection of integral control. That is to say, not only is the change in serum cal-

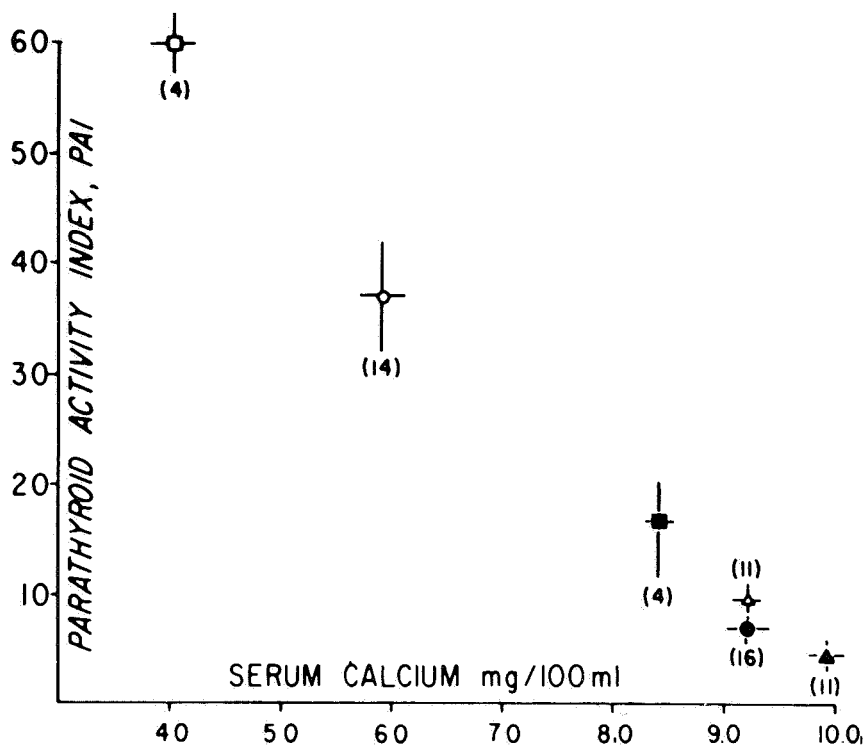


FIGURE 26. The relationship between serum calcium concentration and parathyroid activity index (PAI). Symbols are defined in figure 25. PAI is calculated as the T/S ratio times parathyroid size divided by body weight. This index combines the effect, by any given experimental procedure, on size and activity of the parathyroid and corrects for any difference in body size. [From ref. 45; reprinted by permission of the publisher.]

cium responded to proportionally but the prolonged duration of that change is able to alter the amount of functioning parathyroid cell mass. Thus, by increasing both activity and amount of tissue, we can greatly increase the total parathyroid response. I suppose there must be a limit, perhaps when the parathyroids become larger than the rat, but there appears to be a great capacity for an integral control in the parathyroids.

FREMONT-SMITH: What is your AIB?

RAISZ: Very good. Alpha-aminoisobutyric acid, which is non-metabolized. That is the amino acid transport system largely for glycine. The shocking level is up to about half normal. Normal would be about 1.5 millimoles per liter. Shocking level up to half normal has no consistent steps. Between  $\frac{1}{2}$  normal and  $1\frac{1}{2}$  times

normal shocking level, one has a deep curve relating inversely to shocking concentration.

We still need to obtain data on AIB uptake *in vivo*. I would like to see data on normal secretion *in vivo* which would show, I believe, that the greater secretion changes rapidly; that is, the proportional response to the parathyroid is amplified around the normal calcium level, just as the response of the carotid sinus is amplified around normal blood pressure, but proceeding with greater regulation toward normal.

Assuming for the moment that this is likely to be true about the parathyroid, that will answer the problem of prolonged stimulation.

HOLTZER: As you say, that is really tremendous. Have you checked that out as to the number of cells on the surfaces, for example.

RAISZ: We have shown that low calcium increases RNA synthesis in tissue culture.

HOLTZER: This ought to be DNA; that is the whole point.

RAISZ: We have done no direct DNA studies. With embryonic tissue one gets an increase in mitoses and in the number of cells. We have not been able to get many mitoses in adult rat parathyroids in tissue culture, but we know that this can happen *in vivo* from the studies of Talmage and Toft (ref. 46).

NICHOLS: I think that there is reasonable, although less direct, confirmation of these views in human material.

First, there are people with uremia in whom there are hypertrophied parathyroids which are very large indeed and, by inference, there are more cells present. Evidence is also available from such patients, which suggests that these glands revert toward normal. If a successful kidney transplant, which functions for several months, is achieved, the serum calcium of these patients returns to normal. Indeed, if they have metastatic calcification, this disappears. In the one patient I know of, who has since come to post mortem, the parathyroids were normal (ref. 47).

RAISZ: I would like to point out that the other half of the regulation still requires further study; that is, the opposite effects of thyrocalcitonin. I do think that the same kind of approach can be made to studies of thyrocalcitonin regulation.

COPP: The only thing I would do would be to twist it the other way around. We hope to do the experiment you mentioned, and measure the enhanced parathyroid hormone output by immunoassay during EDTA-induced hypocalcemia.

MCLEAN: Before you go on with thyrocalcitonin, I would like to add another human model to the one Dr. Nichols has mentioned. This is a situation in which there is an adenoma of one of the parathyroids, and the other parathyroid glands are exposed to hypercalcemia over a long period of time. This is being intensively studied now and it

will add a great deal to our understanding of the control of parathyroid secretion. The other parathyroids under that situation quiet down.

FREMONT-SMITH: Atrophy——

MCLEAN: I do not think you can use the word "atrophy." They subside. The surgeon looks at them and he says, "These other glands are atrophied." They are being studied now with the electron microscope and more information is coming out about what is going on over a long period of time so that integral control may be observed.

FREMONT-SMITH: You say they quiet down. A sort of functional atrophy—an atrophy of function.

MCLEAN: They function at a lower level. I doubt that they ever cease functioning completely.

RAISZ: There are substantial data from Dr. Sanford I. Roth of Massachusetts General Hospital, not only on the functional atrophy of the human parathyroid gland but also on the effects of calcium on parathyroid morphology in tissue culture (ref. 43). Dr. Roth has inferred from electron-microscope pictures (we should remember that one can only guess at the sequence of events with such static pictures) that there are cycles of hormone synthesis and secretion in the parathyroid cell. Dr. Roth thinks these cycles are slowed down in high calcium and speeded up in a low calcium environment. This kind of effect can be seen both in tissue cultures of rat parathyroids and in human material.

COPP: Dr. Talmage, would you like to say something before we leave the parathyroids?

TALMAGE: One particular aspect of parathyroid action has bothered me. This aspect was illustrated recently by Harrison and Harrison (ref. 48) in a paper relative to vitamin D effects on calcium absorption by the gut *in vitro*. It was not their study of vitamin D that bothered me. However, in their discussion they assumed that parathyroid action on bone was extremely slow. This assumption was made as if it were a generally accepted fact. I think it is very important for us to emphasize here that parathyroid is a very rapidly acting hormone. It produces effects on bone in minutes, not in hours. We should endeavor to counteract the idea of this slow action which is so prevalent in the literature. Apparently this idea results from the fact that small calcium changes are difficult to identify due to the large pool size of extraosseous calcium.

Unfortunately, Dr. Copp's recent emphasis that thyrocalcitonin is faster acting than parathyroid hormone can add to this misconception. While he is speaking in relative terms, the description of parathyroid hormone in this matter has aided the erroneous concept prevalent in the literature. I believe this is one of the most important aspects of the hormone to be kept in mind.

URIST: Does the rate of the response depend on the age of the animal?

TALMAGE: No.

COPP: I would like to comment on this, since you brought the matter up. I believe parathyroid hormone is fast acting, even in dogs and man, but in these species the effect persists for many hours.

TALMAGE: You are talking about the half-life of the hormone after it is secreted.

COPP: I am not talking about the half-life of the hormone in circulating blood, which Sherwood et al. (ref. 40) have shown to be on the order of 20 to 30 minutes. I am talking of the duration of action, which may be for many hours.

NICHOLS: I think your point is extremely well taken. Part of the reason we think it takes so long is because we have always looked at it from the point of view of injecting parathyroid hormone into intact animals. I think Dr. Arnaud ought to speak about this in relation to his newer animal preparations. Our recent experience, using thyrocalcitonin, suggests that parathyroid hormone effects occur within an hour.

COPP: We have observed elevations in plasma calcium within 20 to 30 minutes of intravenous administration of parathyroid hormone in the dog, and there must be changes in bone which precede this elevation.

TALMAGE: Yes; but one-half hour is a relatively short time compared with 6 hours, as reported by Harrison and Harrison (ref. 48).

COPP: Well, a 6-hour delay is ridiculous.

NICHOLS: Good point. Howard Rasmussen suggested in 1961 (ref. 49) that there are effects of an injection of parathyroid hormone that last for a very long time, these being the ones on the bone mineral. Recently, we have done some experiments with thyroparathyroidectomized animals given a single injection of parathyroid hormone, looking for the effects on acid production by cells and the solubility of the mineral.\* It turns out that there is a clear time sequence of these events as you would suspect; namely, that an effect on the acid production by the cells can be seen within 1 or 2 hours. This has largely faded after 12 to 18 hours; lactate production then falls below normal up to 48 hours and subsequently drifts back to normal. The mineral effect, on the other hand, comes later, lagging anywhere up to 6 to 8 hours behind the changes in acid production and this continues to increase for as long as 2 days.

RAISZ: I have contradictory data. In tissue culture one can show

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\*Asher, J. D.; Nichols, G., Jr.; et al.: Unpublished observations. Some of these observations were conducted as part of a required class laboratory by members of the Harvard Medical School class of 1969.

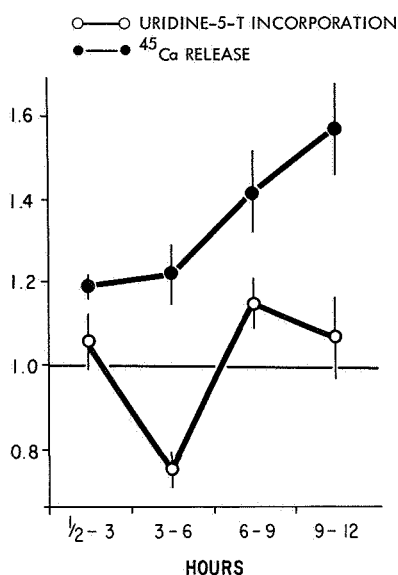


FIGURE 27. Treated versus control ratios plotted against time showing the effect of parathyroid hormone ( $1 \mu\text{g/ml}$ ) on the release of calcium and the incorporation of uridine into RNA in paired cultures of embryonic bones. Each point represents the mean and SE of 8 to 12 pairs of cultures (6 bones per culture). Embryonic bones in which  $^{45}\text{Ca}$  had previously been incorporated were precultured for 24 hours and then transferred to media with or without parathyroid hormone. The relative release of  $^{45}\text{Ca}$  into the medium and uptake of uridine from the medium into the bone RNA were measured for various intervals after hormone addition. [From unpublished observations of L. G. Raisz and Ingrid Niemann.]

an apparently immediate effect on calcium release from embryonic bone. These bones are labeled with  $^{45}\text{Ca}$  and then precultured so that exchangeable  $^{45}\text{Ca}$  on the surface is largely removed. During the first 3 hours after addition of parathyroid hormone, there is a 20-percent increase in calcium release (fig. 27). This represents less than 1 percent of total bone calcium. This release rate continues during the first 6 hours; after 6 hours there is a sharp break in the curve and the calcium release rapidly increases for parathyroid hormone-treated bones. Hence, there is a change from an initial phase of a modest parathyroid effect on calcium release to a second phase of augmented

parathyroid effect. In these experiments we also studied the effect of parathyroid hormone on the incorporation of uridine into bone RNA. During the first 6 hours there was inhibition of uridine incorporation. This may represent the known inhibitory effect of parathyroid hormone on the osteoblasts. At about 6 hours there is an increase in the incorporation of uridine into RNA; this increase is even more marked in experiments where the tissue is pulse labeled for 1 hour (fig. 28).

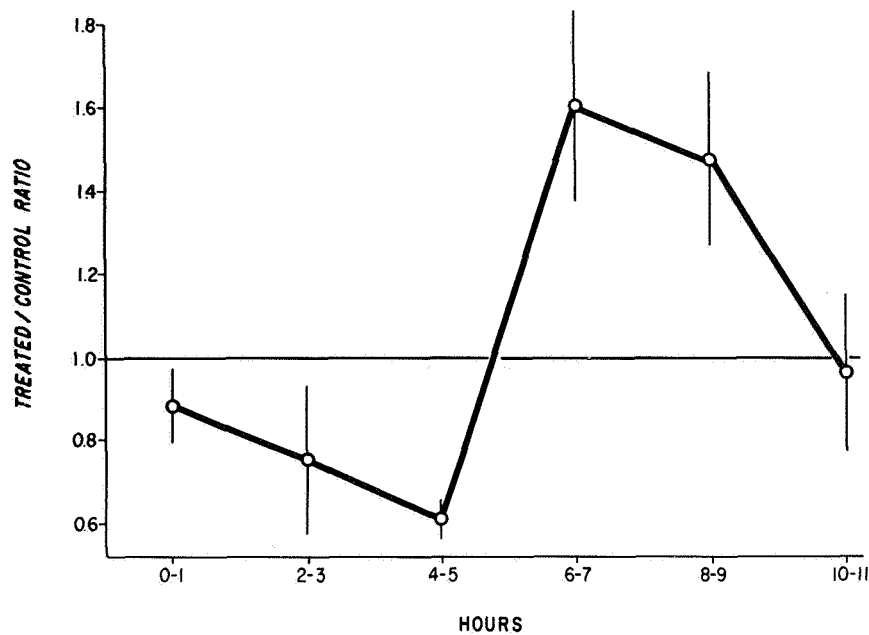


FIGURE 28. Effect of parathyroid hormone ( $1 \mu\text{g}/\text{ml}$ ) on uridine-5-T incorporation into RNA after 1-hour pulse label. Each point represents the mean and SE of six to nine pairs of cultures (two to six bones per culture). Conditions as for figure 27. The 1-hour uridine uptake shows an abrupt increase for the 6- to 7-hour period. [From unpublished observations of L. G. Raisz and Ingrid Niemann.]

SAXÉN: Is this into the total RNA?

RAISZ: Yes. This is preliminary work; we have not been able to measure pool size or to isolate the RNA and characterize it. It does fit in with the idea that parathyroid hormone initially causes a rapid effect on calcium movement in the cells and that, subsequently, the bone responds with a change in its cell population. We can actually see this histologically after 8 to 12 hours in culture with the appearance of more osteoclasts. This then goes on to the extensive complete resorption that one can see in tissue culture.



ROBINSON: Then, is this bone messenger RNA or soluble RNA?

RAISZ: This is total RNA. We think that the 1-hour incorporation is mostly messenger RNA.

ROBINSON: Why not? Another thing is, if it affected the cell right away you would not see the effect for 4 hours, would you? You said it takes the cell that long to "tool up."

RAISZ: You see messenger RNA synthesis right away.

COPP: I would like to ask Dr. Bélanger to comment on this point, since he has some information on the cellular responses in bone to injection of parathyroid hormone. There is an immediate effect and a delayed effect which might correspond to increased osteoclast activity.

NICHOLS: Dr. Copp, Dr. Raisz said earlier that he had completely opposite data. In point of fact, they are not a bit opposite. The early mineral effects that he is seeing occur just when we see an effect on cellular acid production, both lactate and citrate. Later on, about 6 to 8 hours after injection, the passive solubility of the mineral starts to change. If I am not mistaken, this is the point where you start to get a sharp rise in calcium release. What I am saying is that it takes several hours for the surface that we were talking about to become modified by the increased cellular acid production which is induced by PTH. When you use a live tissue in your system, it is reflected by a lag in the calcium release. We get exactly the same thing when using our fragments if we keep them alive.

RAISZ: Having injected the PTE in the animal?

NICHOLS: Yes.

RAISZ: You are saying that there is a calcium effect that you could not measure, but that you could measure the effect on lactate.

NICHOLS: Looking at the steady-state distribution of calcium between medium and bone, there is an effect in the early phases around live pieces of bone. But if you look only at inactivated tissue, the effect does not appear until later (ref. 50). The important point is that the mineral, and its solubility, is modified by the change that has been going on in the metabolism of the cells.

TALMAGE: I would like to say that we have lots of RNA data not only at 1 hour but at 20 minutes, but I have an entirely different interpretation on this RNA. I do not believe this early change has anything to do with homeostasis. I would just like to say that I have these data and would like to discuss them later.

NICHOLS: Dr. Raisz says it is messenger RNA in its early phase. I do not think that is correct. I have reason to believe so, and would like to discuss the point later.

COPP: You can certainly bring up this matter later. One of the important questions is the way in which parathyroid hormone produces

its effect in normal calcium homeostasis. Dr. Bélanger has some views on this.

BÉLANGER: At this stage, I can only offer the following comments related to the present discussion.

If we look at various parts of the skeleton after endogenous stimulation of the parathyroid gland, or after parathyroid hormone, as done by Dr. Copp, we can see this. If we compare one particular region, for instance, in these two fashions, first look at it in its mineralized phase through a modified type of microradiography using alpha bombardment, we can see that the loss in density is coincidental in the various areas of bone.

On the other hand, the loss of organic material is over a wider area than the loss of mineral substance (ref. 51). These data, which we obtained in British Columbia, allowed us to think that the primary effect of parathyroid hormone was on the cells and that the immediate response of the cell was to destroy somehow or to modify the organic substance around some of these cells; the amount of destruction being far greater than the amount of loss of salt seemed to indicate that the organic effect was primary.

COPP: The osteocyte is now threatening the osteoclast.

BÉLANGER: These are two different things, two different worlds indeed.

COPP: Is there any further comment on the parathyroid?

URIST: Dr. Arnaud, your work has been mentioned several times. Do you agree with what has been said about it here?

ARNAUD: I am afraid that both the mechanism of PTH action and bone cell metabolism are exceedingly complex and the techniques available for their study *in vivo* are quite crude. The measurement of an increase in the concentration of calcium in the plasma of the PTH-treated parathyroidectomized organism, although a physiologically important effect, merely represents the end result of a series of reactions of which we know little. Recognizing this, the measurement of the time required to observe this result has been complicated by the fact that most studies have been done in animals whose thyroid glands have been left intact. Under these conditions, thyrocalcitonin secretion is not controlled and its presence might be expected to inhibit the plasma-calcium response to administered PTH. This is in fact the case. When the thyroparathyroidectomized rat is given parathyroid hormone a response can be observed as early as 30 minutes and possibly sooner. I should expect that if one could measure another specific, and more sensitive, index of parathyroid hormone action on bone, an almost instantaneous effect could be observed.

COPP: I would like to ask one question. Do you consider half an hour fast or slow?

ARNAUD: I do not think that we have the techniques to measure how rapidly parathyroid hormone acts.

COPP: It has been known for a long time that a response may occur within 20 to 30 minutes.

ARNAUD: Well, we are measuring the end result.

BAUER: Does the parathyroid hormone act in the absence of vitamin D?

ARNAUD: We have made our position rather clear about this. Our studies have shown, first, that it is extremely difficult to produce an animal which is entirely depleted of vitamin D; and second, that the osseous response, if any, of this animal to parathyroid hormone depends upon the degree of vitamin D deficiency induced (refs. 52 to 54). Harrison et al. (ref. 55) and Harrison and Harrison (ref. 56) were the first to demonstrate this dependency relationship. There has been work reported which is contradictory (refs. 57 and 58), but the major portion of it can be questioned on the basis of the adequacy of vitamin D depletion.

The important concept that has come out of our studies of vitamin D deficiency and its interrelationship with parathyroid hormone action is that parathyroid hormone appears to have its characteristic effect on the renal tubule in the vitamin-D-deficient animal, whereas it does not have its characteristic effect, or at least the degree of effect, on bone.

This is interesting in that studies done by Rasmussen et al. (ref. 59) and by Tashjian et al. (ref. 60) with inhibitors of protein synthesis seem to be analogous to the results observed in vitamin D deficiency. The influence of parathyroid hormone on the kidney of the actinomycin-D-treated and the vitamin-D-deficient rat is qualitatively the same as that of the normal animal, whereas its effect on bone is, in large part, blocked.

TALMAGE: I would like to disagree with that for the record. Actinomycin D does not specifically block parathyroid action in bone, at least relative to the control of plasma-calcium levels. It is true that this drug drops calcium levels and appears to inhibit the action of injected hormone, but this is a result of the general effect of this drug on all cells. We feel that we have demonstrated convincingly that the hormone is still functioning in animals under heavy actinomycin D treatment.

COPP: If we can continue this discussion later, I would now like to complete the endocrine contribution to calcium homeostasis.

We will now turn our attention to the thyroid, the other endocrine gland directly involved in calcium homeostasis. In our original experiments (ref. 61) in which we perfused the thyroid-parathyroid apparatus in the dog with high and low calcium blood, we observed that exposure of the glands to hypercalcemia resulted in a prompt fall in systemic plasma calcium which was much more rapid than the fall

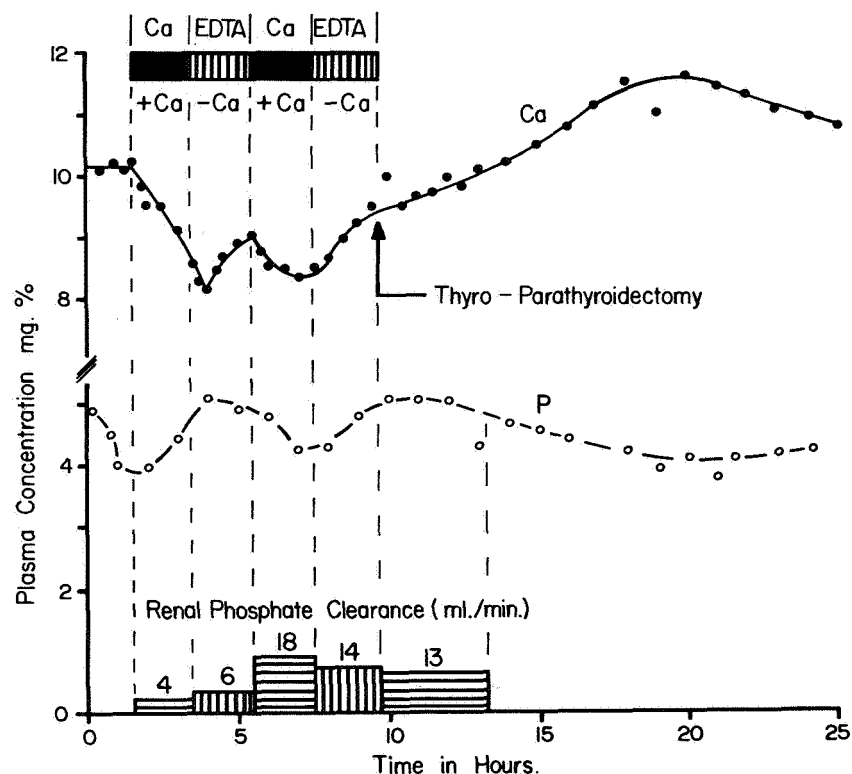


FIGURE 29. Changes in plasma calcium during successive perfusions of the thyroid-parathyroid glands in a fasting dog with blood alternately high and low in calcium. The glands were removed as indicated by the arrow at the end of the last EDTA infusion. [Adapted from ref. 42; reprinted by permission of the publisher.]

which occurred after surgical removal of the glands. In the definitive experiment illustrated in figure 29, high calcium perfusions of the glands caused a rapid fall in blood calcium, while low calcium (EDTA) perfusions caused a rise, presumably due to the release of endogenous parathyroid hormone (ref. 62). To test the current hypothesis that the effect of hypercalcemia was due to suppression of parathyroid hormone production, after establishing the responses through two cycles, we removed the thyroid and parathyroid glands. Instead of the anticipated fall, the plasma calcium rose and remained elevated for many hours. It was evident that the effect of hypercalcemia was a result of the release of a humoral agent which was responsible for the rapid fall in systemic blood calcium. Indeed, we found that the high calcium perfusates did produce hypocalcemia when injected into a second dog. These results were confirmed by Kumar et al. (ref. 63), by using ion-exchange resins to modify the calcium concentration in the perfusing

blood. We named this new hormone "calcitonin," since it appeared to be involved in regulating the level, or tone, of calcium in body fluids. While first thought to come from the parathyroids, it is now apparent that the calcitonin originally demonstrated in these experiments was of thyroid origin (ref. 64). In 1963, Hirsch et al. (ref. 65) observed that destruction of the parathyroids by hot-wire cautery caused a much greater fall in plasma calcium than that which occurred after surgical removal of the glands. They were also able to extract a potent hypocalcemic and hypophosphatemic substance from thyroid tissue, to which they gave the name "thyrocalcitonin" to indicate the gland of origin, and its possible identity with calcitonin. Care (ref. 66) has clearly demonstrated release of thyrocalcitonin by high calcium perfusion of pig thyroid, which contains no parathyroid tissue. (See fig. 30.) In collaboration with Dr. Bélanger, we have also shown that 24 hours of mild hypercalcemia (+ 10 to 15 mg/100 ml) in the sheep results in hypertrophy of thyroid cells—particularly the parafollicular light cells.

NICHOLS: May I ask if those changes were in both the parafollicular and the follicular cells?

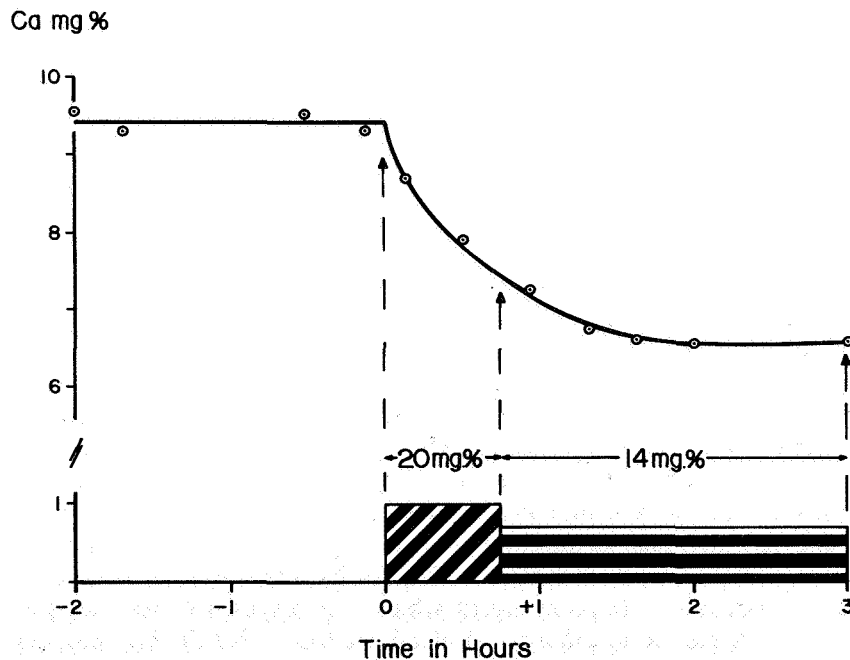


FIGURE 30. Changes in systemic plasma calcium associated with high calcium perfusion of the pig thyroid *in situ*. The calcium concentration in the perfusing blood is indicated. [From data in ref. 66; reprinted by permission of the author.]

COPP: Dr. Bélanger should comment on this point. All thyroid cells appeared larger, but the effect was most marked in the parafollicular cells. Dr. W. Chase prepared electron micrographs which showed increased activity in the endoplasmic reticulum of these cells.

BÉLANGER: Of course, these are old names which date back to the 1920's. It is now possible to demonstrate that these are two aspects of the same cell, and that the parafollicular cells, which are not parafollicular at all, are away from the follicle and are distal to the colloid. These are young cells that are possibly capable of responding to STH-stimulation but not to TSH, and are capable of manufacturing thyrocalcitonin but not thyroid hormone as we understand it.

Now, as these cells mature and move toward the colloid to replace the cells that are dying out there, they also acquire the ability to manufacture thyroid hormone. Therefore, in our estimation, all types of cells can make thyrocalcitonin, but only the more adult, or more mature, cells can make thyroid hormone.

PECK: Did you find any evidence for increased thyroxine genesis?

COPP: With calcium?

PECK: Yes.

COPP: No. That would be an interesting experiment but we have not done it yet.

BÉLANGER: There is histochemical evidence to the effect that the production of thyroid proteolytic enzyme, which is associated with the utilization of thyroglobulin, actually is stimulated by treatment such as Dr. Copp has described; that evidence would indicate that both functions are enhanced.

COPP: Both Dr. Bélanger and we have observed some thyroid hyperplasia in rats made hypercalcemic by feeding a high calcium, low phosphate diet. However, the effects were not as striking as in the sheep.

NICHOLS: Do you think the calcitonin is stored with the colloid, or is this very rapid change a secretory phenomenon?

HEANEY: Apparently there is a vast difference in the amount of colloid. It seems inconceivable to me that an equivalent amount of thyroid hormone could have been secreted under the conditions that would not have affected——

BÉLANGER: I do not know about the physiologic detection, but certainly since the proteolytic enzyme production is also increased, it seems that as the utilization of this thyroid hormone is increased, then the residual amount of colloid could very well not be representative of the amount of production of the hormone, because the turnover rate also appears to be considerably greater.

COPP: We should do this experiment sometime. It would be very interesting to see the effect on protein-bound iodine (PBI).

TALMAGE: Two years ago, when we made our first report concerning the presence of thyrocalcitonin in rats, we noted that the release of thyrocalcitonin which followed parathyroidectomy was also accompanied by a very marked increase in  $^{131}\text{I}$ -PBI (ref. 67).

COPP: I would now like to turn the meeting over to Dr. Arnaud so that he can tell us of the very exciting work in his laboratory on the chemistry and secretion of thyrocalcitonin.

ARNAUD: Thank you, Dr. Copp. I should like to present the work which Drs. Tenenhouse, Rasmussen, and I have done on the isolation and characterization of porcine thyrocalcitonin (ref. 68) and also discuss some preliminary studies of a radioimmunologic assay system for the polypeptide that Dr. Littledyke and I have developed. All presently available evidence indicates that this polypeptide is the hormone thyrocalcitonin; but it is important to point out that Baghdiantz et al. (ref. 69) have reported a purified preparation of thyrocalcitonin that may have a much smaller molecular weight. Further studies are

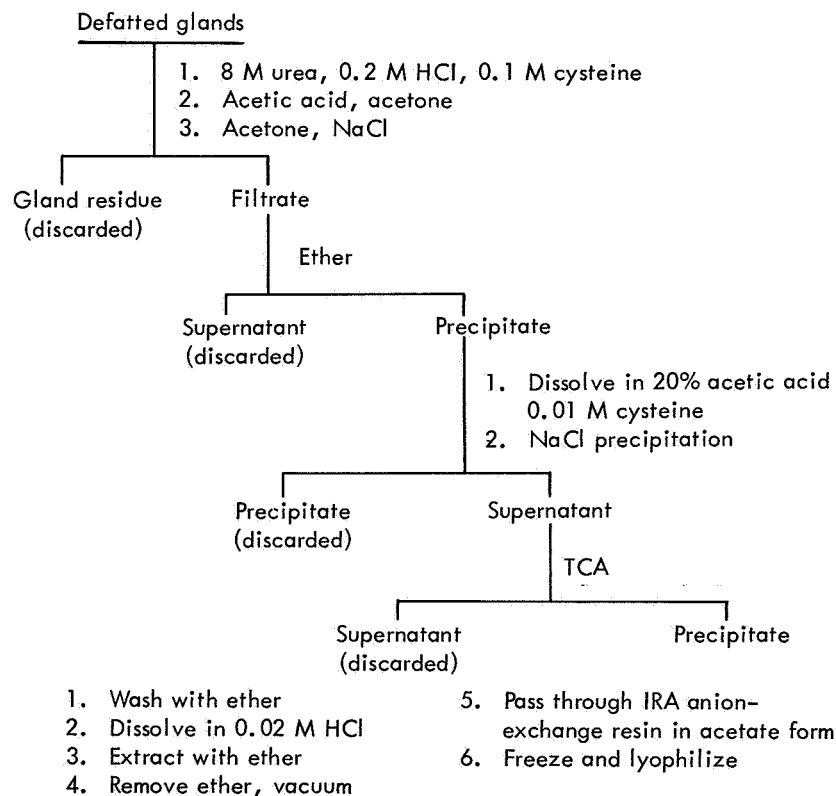


FIGURE 31. Flow diagram of the extraction of thyrocalcitonin from dried, defatted, fresh, frozen, porcine thyroid glands. Final product is a crude trichloroacetic acid (TCA) powder.

required before the possible relationships between these two products can be known.

As shown in figure 31, dried and defatted, fresh, frozen porcine thyroid glands are carried through urea-HCl-cysteine extraction and solvent, salt and trichloroacetic acid (TCA) precipitation procedures resulting in a crude powder extract. This procedure is almost identical with that applied to bovine parathyroid glands in our purification procedure for parathyroid hormone (ref. 70). This crude powder extract is relatively stable when refrigerated in the lyophilized state at 4° C and produces a decrease in the plasma calcium of calcium-deficient rats of 1.5 mg/100 ml at an intravenous dose of 30 to 40 micrograms of protein (ref. 71) 1.5 hours after administration. This crude powder is further purified by dextran gel chromatography on columns of Sephadex G-75 using 0.2 M ammonium acetate, pH 4.6 as eluant. The eluted material is pooled and lyophilized, as shown in figure 32. Although considerable biologic activity is observed in both pools, tubes 92 to 100 and tubes 100 to 120, the greatest was present in the former, and it was chosen for further characterization.

When this material is subjected to either starch gel (fig. 33) or polyacrylamide (fig. 34) electrophoresis, it migrates as a single component.

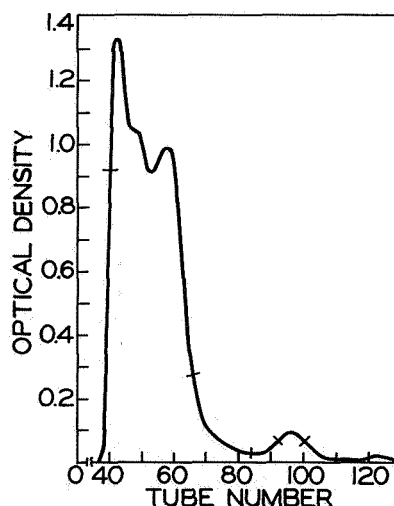


FIGURE 32. Gel filtration of crude thyroid extract (see fig. 31) on Sephadex G-75 column 2.5 times 130 centimeters. The eluant was 0.2 M ammonium acetate, pH 4.6. The peak in tubes 92 to 102 contained the major portion of the biologic activity. [From tenenhouse et al., ref. 68.]





FIGURE 33. The patterns obtained on starch gel electrophoresis when crude and purified preparations of thyrocalcitonin were analyzed. Crude TCA powder (left), active fraction from gel filtration on G-100 (middle), and active fraction from gel filtration on Sephadex G-75 (right). The material on the right is the substance which was further characterized. [From Tenenhouse et al., ref. 68.]

It has a molecular weight of approximately 9000, and amino acid analyses (table I) show that it is unique in that it has a single, half-cystine residue.

HEANEY: Dr. Arnaud, in virtue of our past discussions, are there any iodinated amino acids in that molecule?

ARNAUD: I will tell you about an interesting finding. The —SH group of the cystine residue is not titrable, so that it is not free, at least not by any criteria that we have been able to determine. It is possible that an iodine molecule might be linked with this —SH group; we have not been able to look into this yet. But, it is important to remember one thing in this regard. The hormone-extraction procedure involves the addition of cysteine at two separate points; it is possible that the molecule might bind this amino acid so that the final product contains a cysteine residue in nonpeptide linkage.

We have been able to produce antibodies to this purified material by conjugating it with carbodiimide (ref. 72) to rabbit albumin and by injecting the conjugated material into rabbits with Freund's complete adjuvant. Figure 35 is a gel diffusion pattern in which we have placed the antibody in the center well (IV), a homogenate of porcine thyroid tissue in well III, the crude TCA extract in well I, and the purified material in well II. Therefore, the antibody is quite specific for the isolated peptide in that lines of identity are present.

Figure 36 is a microimmunoelectrophoretic study of the crude TCA extract (I) and purified preparation (II) with antibody in the longitudinal center well (IV). There is a single band of precipitation where

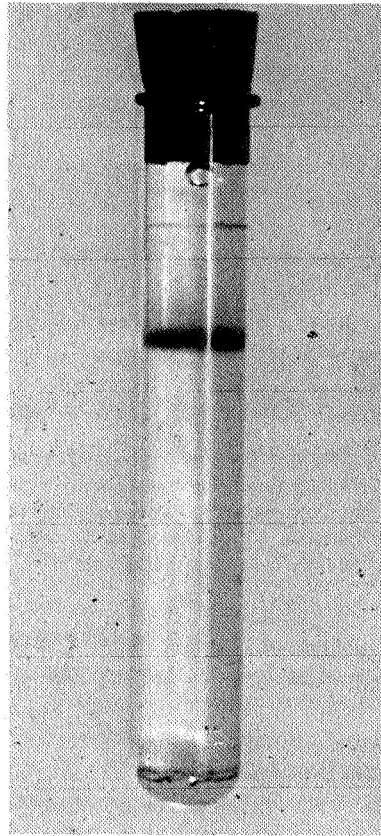


FIGURE 34. Pattern obtained on polyacrylamide disk electrophoresis of the active fraction from gel filtration on Sephadex G-75. Sample size was 100 micrograms.

antibody and antigen have reacted, which again supports the idea that the prepared antibody is specific for the isolated polypeptide.

We decided that we would like to find out which cells of the thyroid gland produce this polypeptide. With Gary Hargis and Gerald Williams in Chicago, we carried out a series of immunofluorescent studies of the thyroid gland with the antibody conjugated to the fluorescent material, lissamine rhodamine B 200 (ref. 73). We see (fig. 37) fluorescence over the cytoplasmic material of all the thyroid cells of the pig. The thyroid glands of the dog, rat, and human have been studied in a similar manner with similar results. Notice that there is no specific fluorescence over any of the colloid.

PECK: Can you block that fluorescence?

TABLE I  
AMINO ACID COMPOSITION OF THYROCALCITONIN

Amino acid	Residues per molecule			
	22 hours	72 hours	22 hours oxidized	Calculated
Cysteic acid.....	—	—	1.07	1
Aspartic acid.....	7.06	6.86	—	7
Threonine.....	3.69	3.26	3.95	4
Serine.....	3.76	3.00	3.48	4
Glutamic acid.....	9.72	9.25	—	10
Proline.....	4.36	4.86	—	4
Glycine.....	5.22	5.06	5.42	5
Alanine.....	7.82	7.83	7.95	8
Valine.....	4.24	4.20	4.25	4
Cystine.....	.50	—	—	.50
Methionine.....	.90	.67	—	1
Isoleucine.....	2.79	2.76	2.84	3
Leucine.....	9.15	8.96	9.27	9
Tyrosine.....	2.22	2.00	—	2
Phenylalanine.....	2.13	2.20	1.99	2
NH <sub>3</sub> .....	8.03	10.16	—	7
Lysine.....	8.38	8.29	8.08	8
Histidine.....	1.22	1.27	1.02	1
Arginine.....	3.60	3.48	3.62	4
Tryptophan.....	1.00	—	—	1

ARNAUD: Yes. It is specifically blocked in two different ways. If we overlay the tissue with nonfluorescinated antibody, fluorescence is totally blocked. Similarly, incubation of the fluorescinated antibody with the isolated polypeptide overnight at 4° C before overlaying the tissue results in the complete absence of fluorescence.

We became intrigued with the idea that parathyroid hormone and thyrocalcitonin might be chemically similar because of their similar molecular weights. If one lines up the two molecules in terms of their amino acid residues (table II), they compare rather closely. There are some important differences, however. Of particular note is the presence of three histidine residues in parathyroid hormone, compared with only one in the thyrocalcitonin peptide, and the absence of cysteine in parathyroid hormone. Further, when one elutes these polypeptides from carboxymethyl cellulose (CMC) columns with identical concentration gradients of ammonium acetate (fig. 38), parathyroid hormone comes off at a concentration of 0.33 *M* and the thyrocalcitonin peptide at 0.1 *M*, indicating that parathyroid hormone is a more highly charged molecule.

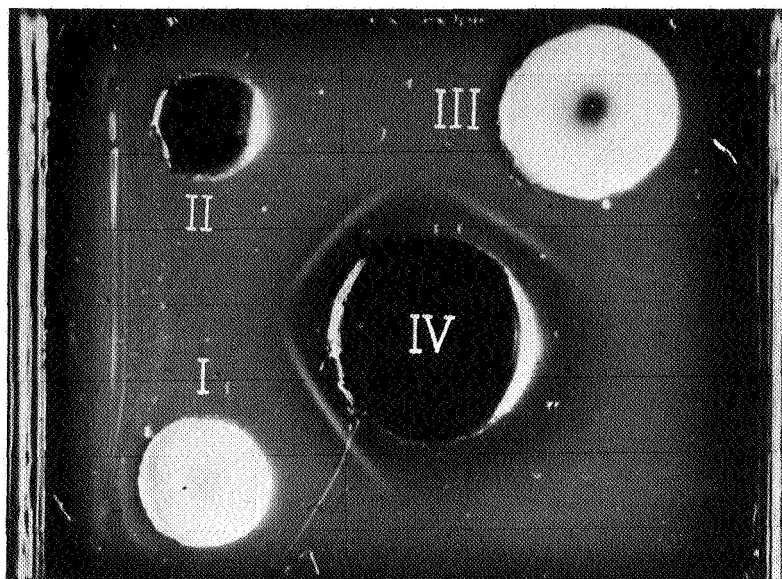


FIGURE 35. Microimmunodiffusion pattern of reaction between antiserum to TCT and three preparations of TCT, showing pattern of fusion. I, crude porcine TCT extract; II, active fraction from G-75 fractionation; III, homogenate of porcine thyroid tissue; IV, rabbit antiserum to material in II. [From Hargis et al., ref. 73; reprinted by permission of the publisher.]

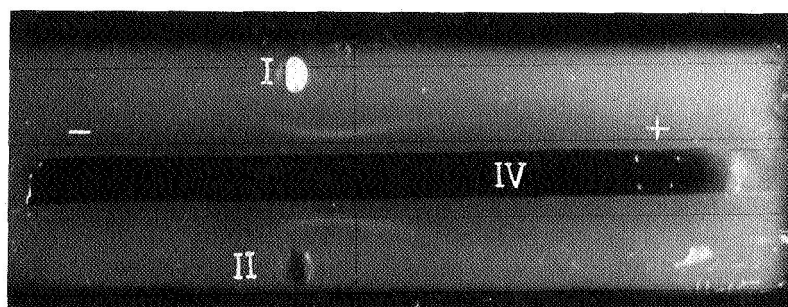


FIGURE 36. Microimmunoelectrophoresis pattern of reaction between antiserum to TCT and two preparations of TCT, showing single corresponding arcs in anodic position (barbital buffer, pH 8.8). I, crude TCA extract; II, active fraction from fractionation on G-75; IV, rabbit antiserum to II. [From Hargis et al., ref. 73; reprinted by permission of the publisher.]

Dr. Littledike, Miss Tsao, and I have developed a radioimmunologic procedure for the assay of this polypeptide in biologic fluids, which is similar to that described by Yalow and Berson (ref. 74) for insulin.

The radioimmunoassay depends on the competitive inhibition of stable thyrocalcitonin with  $^{131}\text{I}$ -labeled thyrocalcitonin for antibody binding, and the degree to which stable thyrocalcitonin will inhibit

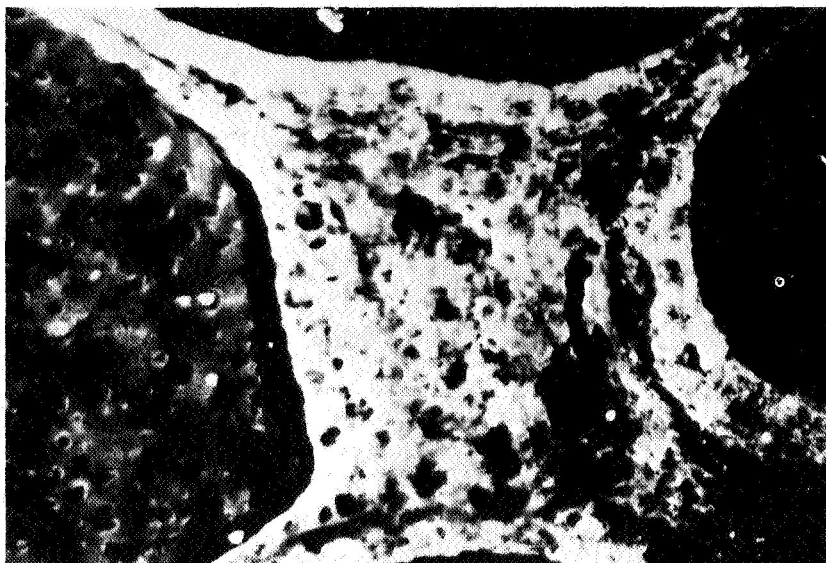


FIGURE 37. Porcine thyroid gland: fluorescent antibody globulin to TCT stain, showing fluorescence confined to thyroid epithelial cells. 400  $\times$ . [From Hargis et al., ref. 73; reprinted by permission of the publisher.]

TABLE II

COMPARISON OF THYROCALCITONIN AND PARATHYROID HORMONE IN AMINO ACID COMPOSITION

	Thyrocalcitonin	Parathyroid hormone
Aspartic.....	7	8
Threonine.....	4	1
Serine.....	4	6
Glutamic acid.....	10	10
Proline.....	4	2
Glycine.....	5	4
Alanine.....	8	6
Valine.....	4	7
Cystine.....	.5	0
Methionine.....	1	2
Isoleucine.....	3	3
Leucine.....	9	7
Tyrosine.....	2	1
Phenylalanine.....	2	2
NH <sub>3</sub> .....	7	7
Lysine.....	8	7
Histidine.....	1	3
Arginine.....	4	4
Tryptophan.....	1	1

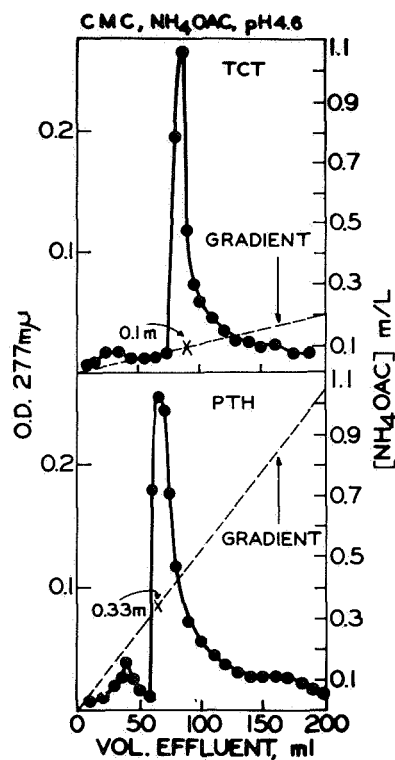


FIGURE 38. Column chromatography of porcine TCT polypeptide and purified parathyroid hormone (PTH) on carboxymethyl cellulose using increasing ionic strength gradient, ammonium acetate, pH 5.0. Major protein peak of PTH elutes at 0.33 *M* and that of TCT at 0.1 *M*.

iodinated thyrocalcitonin from being bound to antibodies is proportional to the concentration of stable thyrocalcitonin added to the mixtures.

Antibody-bound  $^{131}\text{I}$ -labeled peptide is separated from free  $^{131}\text{I}$ -labeled peptide by a chromatoelectrophoretic technique on Whatman 3 MC filter paper strips and subsequently evaluated by means of a chromatogram strip counter and peak area integration. Figure 39 shows a series of tracings obtained when progressively increasing quantities of stable peptide are added to mixtures containing the same amount of antiserum (1 to 50 000 dilution) and labeled peptide after 4 days' incubation at 4° C. As you can see, increasing the concentration of stable peptide is associated with a decrease in the quantity of labeled peptide bound to antibody.

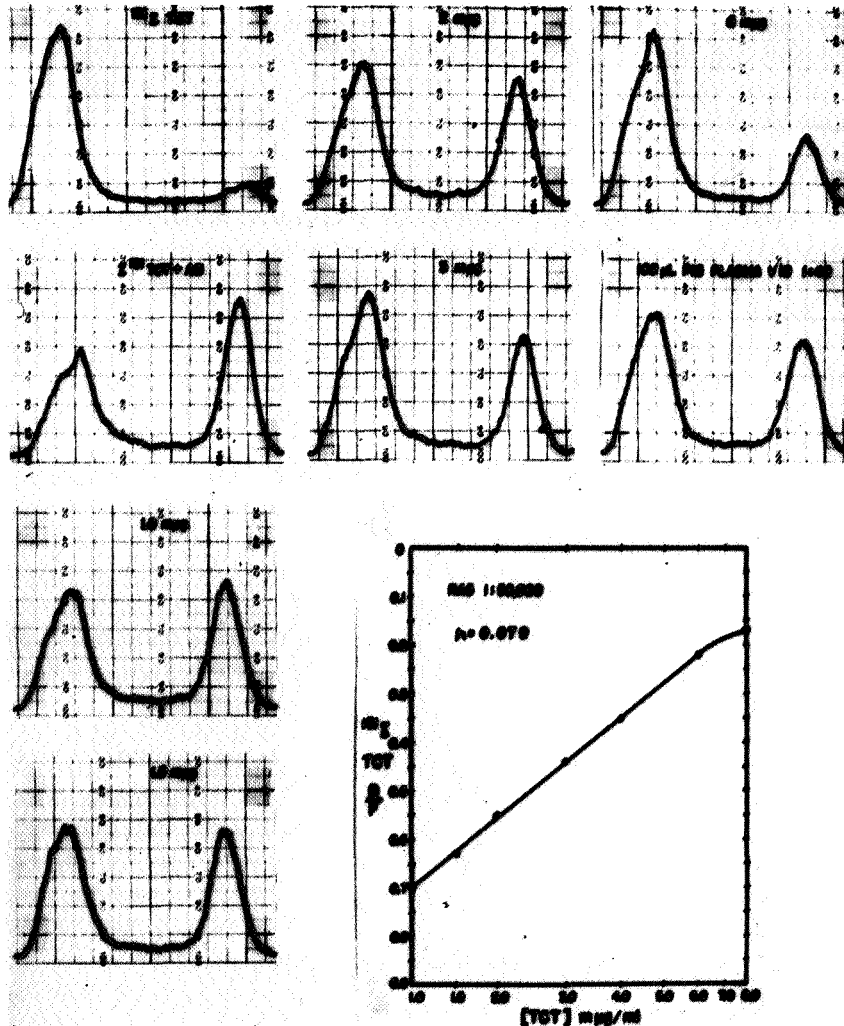


FIGURE 39. Scans of radioactivity on paper-strip chromatoelectrophoretograms of mixtures of  $^{131}\text{I}$ -labeled porcine thyrocalcitonin (TCT) and rabbit antiporcine TCT antiserum (dilution 1 to 50 000) in the same concentration but with varying concentrations of unlabeled porcine TCT as indicated. The right of the two peaks on each tracing represents migrating antibody bound, and the one on the left, free  $^{131}\text{I}$ -labeled peptide which remains at the origin. The tracing marked " $^{131}\text{I}$  TCT" is a scan of a mixture to which no antiserum had been added and the small migrating peak "damaged"  $^{131}\text{I}$  TCT. No unlabeled-TCT was added to the mixture represented by the tracing marked " $^{131}\text{I}$  TCT + AB." The standard curve (right) was obtained by measuring the areas under the bound and free peaks of each scan, expressing them as ratios (B/F) and plotting this value against the logs of the corresponding known concentrations of added, unlabeled TCT. The scan just above the standard curve was obtained when the peripheral plasma of a calcium loaded intact young pig was substituted in a dilution of 1 to 40 for unlabeled TCT in the mixture. Its concentration was calculated as 500  $\mu\text{g}/\text{ml}$  of whole plasma.

If one plots the ratios of the areas under these curves (the ratio of the antibody bound to free labeled peptide) against the log of the concentration of added stable peptide, one obtains a straight line. To measure the concentration of peptide in an unknown solution, one merely adds an aliquot to the mixture of antibody and labeled peptide in place of the standard stable peptide. The assay can easily detect 1.0 m $\mu$ g of peptide per milliliter of incubation mixture. Greater sensitivity can be achieved but it is not necessary because the concentrations in normal plasma are high (young pig, 100 to 400 m $\mu$ g/ml). In nine consecutive assays, the index of precision ( $\lambda$ ) was 0.079.

The concentration of antibody is critical. If too much is added, the competition between labeled and stable peptide for antibody binding becomes difficult to demonstrate, and the sensitivity of the assay suffers.

Using this radioimmunoassay as a sensitive tool, we sought to determine if there was any immunologic similarity between highly purified bovine parathyroid hormone and the thyrocalcitonin molecule. To our surprise, minimal crossreactivity could be demonstrated.<sup>1</sup>

Our attempts at radioimmunoassay in the beginning were quite crude, and my attempts during the past 6 months have been primarily to improve the selection of antisera and to look at thyrocalcitonin from other species.

Since my background is internal medicine, I was interested to see if a polypeptide similar to the one we isolated from porcine thyroid glands was present in human plasma. Figure 40 is from a study made using serial dilutions of my plasma in the immunoassay system. The value for the bound-to-free ratio obtained with my undiluted plasma falls directly on the curve obtained with standard porcine peptide; with serial dilution, an almost identical set of points is observed. This indicates that an immunologically similar polypeptide circulates in human plasma, and it can be precisely measured in terms of porcine peptide equivalents.

We have not really gone very far in measuring the hormone in disease states. All we can say is that it circulates in substantial quantities. We have obtained from 25 normal sera, or relatively normal sera, an average of 30 to 50 m $\mu$ g/ml. This is on the order of 50 times the level of parathyroid hormone which circulates in bovine plasma (ref. 40).

URIST: Have you seen a patient with idiopathic hypoparathyroidism?

ARNAUD: I have measured the polypeptide in the plasma of these individuals, and I do not think it is increased; but I would not like to go on record.

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<sup>1</sup> Exhaustive studies now indicate that the presence of crossreactivity was a result of glassware contamination.



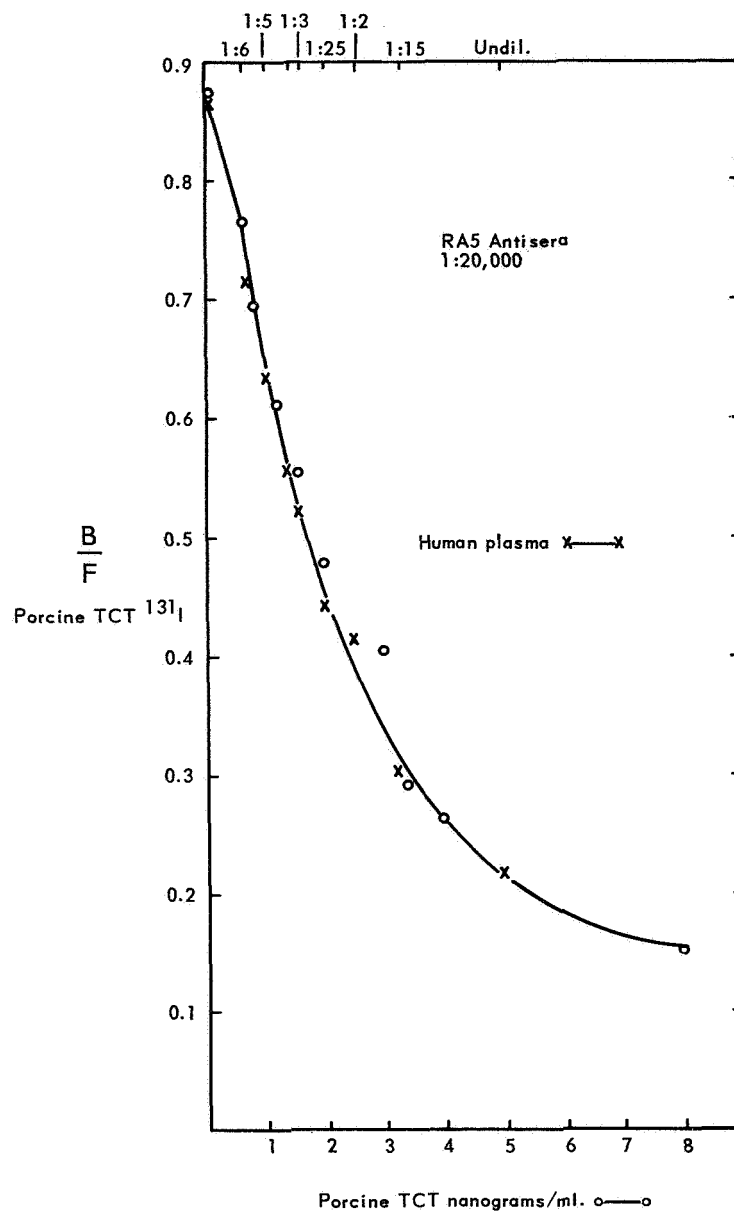


FIGURE 40. Plot of the ratio of antibody bound to free  $^{131}\text{I}$  porcine TCT peptide against the concentration of added stable porcine TCT peptide ( $\times$ — $\times$ ) and human plasma in various dilutions ( $\circ$ — $\circ$ ). Similarity of curves indicates that a substance which circulates in human plasma is immunologically similar to the added porcine TCT peptide.

WALSER: Have you done a thyroidectomized subject?

ARNAUD: Only thyroidectomized pigs. It is much lower, but quite consistently present in all of the animals thus far studied. It is possible that this is due to ancillary thyroid tissue, but this finding concerns us very much and has led us to consider alternative possibilities, such as, that the polypeptide that we isolated may be produced by tissues other than the thyroid gland or that there may be a carrier of the hormone (thyrocalcitonin).

COPP: Have you measured this in osteoporotic patients?

ARNAUD: No; we have not. I have been able to measure thyrocalcitonin in human plasma for 4 weeks now.

NICHOLS: I would like to know where thyrocalcitonin works in the bone.

RAISZ: According to figure 22, thyrocalcitonin acts to inhibit bone resorption. There is some preliminary evidence that this is the way thyrocalcitonin works *in vivo*. I think Dr. Talmage has evidence on this point. The evidence for inhibition of bone resorption in tissue culture is fairly clear. We measure the release of  $^{45}\text{Ca}$  from prelabeled bone in tissue culture into a medium which contains a large amount of stable calcium, so that major changes in this release can be achieved only by affecting resorption. Changes in deposition of calcium would take calcium largely from the unlabeled pool and very little  $^{45}\text{Ca}$  would be returned to the bone. Our experiments (ref. 75) show that in tissue cultures with and without parathyroid hormone, the release of radio-calcium is largely inhibited by the action of thyrocalcitonin (table III).

TABLE III

EFFECTS OF A CRUDE THYROCALCITONIN PREPARATION (TC-J) AND PARATHYROID HORMONE (PTH) ON  $^{45}\text{Ca}$  RELEASE FROM EMBRYONIC BONES <sup>a</sup>

	Dose		
	3 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	30 $\mu\text{g/ml}$
TC-J.....	910	700	770
Control.....	920	920	1050
Difference.....	10 $\pm$ 64	<sup>a</sup> 220 $\pm$ 55	<sup>a</sup> 280 $\pm$ 80
TC-J + PTH.....	1510	780	890
PTH.....	1880	1580	1750
Difference.....	370 $\pm$ 180	<sup>a</sup> 800 $\pm$ 60	<sup>a</sup> 860 $\pm$ 180

<sup>a</sup> Difference significant by *t* test,  $p < 0.02$ . Values, expressed as cpm/0.1 ml medium, are means for  $^{45}\text{Ca}$  release for 3 or 4 bones during 48-hour culture, and mean  $\pm$  SE for the difference between paired bones. [Adapted from Friedman and Raisz, ref. 75.]

Since this experiment was done we have more refined materials, and a partially purified rat thyrocalcitonin has been prepared which is effective in doses of as little as 0.1  $\mu\text{g/ml}$ . When parathyroid hormone is added to the culture, calcium release is increased in bones that are not treated with thyrocalcitonin; but the entire parathyroid effect is brought back to nonresorbing levels by thyrocalcitonin. In addition, thyrocalcitonin inhibits bone resorption in the absence of parathyroid hormone; i.e., what we call control bone resorption.

BÉLANGER: There are some other experiments that have a bearing on this matter. These are the experiments, done immediately before this conference, in which we have evidence that the thyroid gland has been stimulated into taking up the kind of aspect that Dr. Copp has shown. We did this in rats, and then looked at the bones and observed a thyrocalcitonin effect; but it is some sort of an effect that is inhibitory under, let us say, thyrocalcitonin production-like conditions.

There are areas of the bone where the cells become enlarged and are actually surrounded by areas of matrix in which the staining properties will change; for example, under these conditions the usual color with the use of Wright's stain will change to an azure staining.

Now, by comparing this type of staining with a known stain for metachromasia, such as methylene blue, the same areas in the vicinity of the large cell show some metachromasia. This is generally considered as indicative of the presence of some mucopolysaccharides.

In the tibias from rats on a diet low in phosphate, we observed a complete disappearance of these zones of metachromasia except where there was a remnant of cartilage. There are no more of these hypertrophic osteocytes; the osteocytes now appear to be small, and some of them have even died. If you look in other areas more reactive than the tibia, such as the alveolar bone between the molars, we can see that some of these cells, after 7 days of this type of treatment, become totally degenerated. I suggest at this point that if this is indicative of a thyrocalcitonin effect, then the thyrocalcitonin effect is manifested by an inhibition of the metabolism of the large osteocytes which preside over osteolysis.

URIST: What is the target of thyrocalcitonin? Dr. Bélanger, do you suggest that calcitonin controls the process of osteolysis?

BÉLANGER: No, not quite. Osteolysis is the end product, but I think there is some sort of change in the metabolism of the osteocyte whereby it cannot perform its normal osteolytic role—particularly in the large, mature osteocyte which is actually responsible for osteolysis. The osteolytic role may be due to the production of some acids, as Dr. Nichols has mentioned, or perhaps due to the production of some enzymes that break down the organic matrix of the bone. These

effects, then, do not take place, and the cells appear either to remain small or become small. I do not know at the moment.

URIST: Is the osteocyte a target?

BÉLANGER: Yes. This is what these experiments seem to indicate.

URIST: Do you agree with Dr. Raisz, that the osteoclastic activity is inhibited by thyrocalcitonin?

TALMAGE: He did not say. He just said bone resorption. I do not think that we know which cell is responsible.

RAISZ: I only said bone resorption. The response that I showed earlier to parathyroid hormone in the first 3 hours, during which no cellular changes can be seen, is also inhibited by thyrocalcitonin.

BÉLANGER: Osteoclasts are nowhere around.

TALMAGE: I think it is important to emphasize that thyrocalcitonin is just as active in parathyroidectomized animals as it is in control animals. In our experimental system, we place the animal under a calcium stress by our peritoneal lavage system. By this method calcium is removed from the animal at a maximum rate; in the animals maintained on a calcium-free diet, this calcium must be supplied by bone. By such a system, we are magnifying the progress of bone resorption and minimizing bone accretion. If the animals are given thyrocalcitonin during this process, the rate of calcium removal is diminished equally in both control and parathyroidectomized animals. The only difference is the length of time in which the effect lasts. In the animal with intact parathyroids, it reaches its peak effect in 1 hour and returns to normal in 2 or 3 hours. Without parathyroids, it reaches its peak in 2 or 3 hours and may not return to normal for 5 or 6 hours.

The point should be made that phosphate drops proportionately with calcium. Another interesting point pertains to the removal of radioisotopes of calcium and phosphate administered 18 hours or 3 weeks prior to treatment. Thyrocalcitonin caused a reduction in the removal rate during peritoneal lavage with a calcium-free rinse. This reduction was seen in all animals. In parathyroidectomized animals, only long-term administered radioactivity is affected. Because of this difference we do not feel that the two hormones, while producing opposite effects, necessarily work at the same loci in bone cells.

COPP: I might point out that both Dr. Munson and Dr. Wase have some indirect evidence that thyrocalcitonin may also enhance the deposition of calcium in bone.

BÉLANGER: That is secondary.

PRITCHARD: I must comment for a moment on the histologic problem of interpreting osteocyte behavior. It seems very odd that the osteocytes farthest from the blood vessels should be the ones to react. I am not happy about the interpretation given. I think the explanation

is that the osteocytes of primary woven bone are different from the osteocytes of secondary lamellar bone.

COPP: I would like Dr. Arnaud to show his experiment demonstrating the control of thyrocalcitonin secretion.

ARNAUD: This is an experiment done in a female pig weighing 55 kilograms (fig. 41). We measured plasma calcium, phosphate, and immunoassayable thyrocalcitonin peptide before and after calcium loading, when the animal was intact and after its thyroid gland had been surgically excised. The maximum for thyrocalcitonin is about 160 m $\mu$ g/ml.

This study was made at a time when the radioimmunoassay was relatively insensitive and normal plasma levels could not be measured. When a calcium load was given to the animal when its thyroid was intact, a modest degree of hypercalcemia was observed. Coincident with the hypercalcemia, there was a precipitous rise in the level of

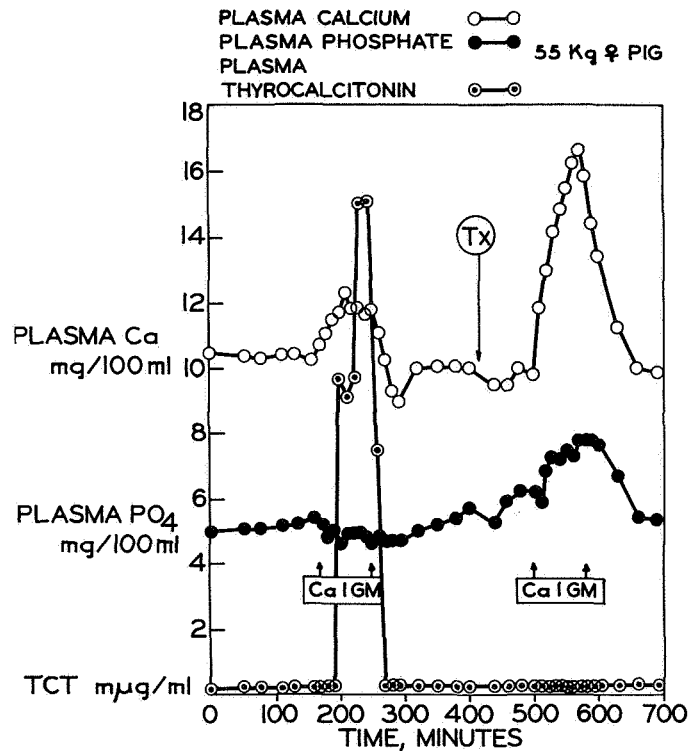


FIGURE 41. Effect of intravenous calcium loading on the concentration of calcium, phosphate, and thyrocalcitonin (TCT) in the arterial plasma of a pig before and after thyroidectomy. TCT levels below 60 m $\mu$ g not measurable at this time in the development of the radioimmunoassay. (Multiply scale for TCT times 10.)

thyrocalcitonin in the plasma. After the calcium loading was discontinued, you can see that there is a relatively rapid return of the plasma calcium to control, perhaps to below control levels, and an immediate decrease in the concentration of thyrocalcitonin to nonmeasurable levels. This occurred over a period of 20 minutes or so; it is a rough estimate of the rate of disappearance of endogenously secreted polypeptide.

In contrast, a markedly different calcium curve is observed when a calcium load is given to the same animal after thyroidectomy. The degree of hypercalcemia that develops is two to three times greater than when the thyroid gland is present, while the level of thyrocalcitonin remains undetectable.

COPP: I would like to thank all of the participants in this session.

## **LOCAL (NONHORMONAL) FACTORS CONTROLLING BONE RECONSTRUCTION**

**Discussion Leader:**

**DR. ROBERT P. HEANEY**

HEANEY: Although I am by nature an endocrinologist, I hope that we will mention hormones in this discussion only to exclude them from further consideration.

I would like to start with a quotation from the book, "Bone," by Dr. McLean and Dr. Urist (ref. 76); a brief paragraph entitled "Local Factors in Resorption" is as follows.

The influence of local factors in the resorption of bone are most easily seen during its growth, when the metaphyses of the long bones are singled out for reconstruction. Such reconstruction invariably includes a considerable amount of osteoclastic resorption, followed by appositional bone formation, with the result that the size and shape of the bones progress toward the adult state. Organization and regulation of these phenomena, affecting most of the bones of the body simultaneously, is a part of overall growth. Some of the factors, at least, are local . . . .

It is these local factors to which we will direct our attention.

I think that although there is more remodeling going on in the skeleton than is characterized by the term "osteon," nonetheless this serves as a suitable temporary focus for some of our discussion. When remodeling occurs, as we all know, a space is tunneled out by osteoclasts, and it is only after this space has been created that new haversian bone is laid down within it. The localization of this space and its polarization in three dimensions are obviously not controlled by hormones.

FREMONT-SMITH: Is that haversian canal a very straight line?

HEANEY: It tends to spiral.

FREMONT-SMITH: To spiral?

HEANEY: A very gradual spiral, mostly longitudinal.

FREMONT-SMITH: Of about what length? A few millimeters, or is it longer?

MCLEAN: Cohen and Harris (ref. 77) did a series of reconstructions—wax models—from serial sections through bone, and they outlined the dimensions and the form of these canals quite accurately. This is the one standard we can look at. A single osteon may be as much as 2 millimeters in length.

FREMONT-SMITH: Thank you.

HEANEY: Although parathyroid hormone, cortisol, growth hormone, thyroid hormone, and a variety of others may well have some effect on the activity of the cells carrying out these processes, obviously the hormone levels are inadequate to explain both the localization and the polarization of these cellular activities, so let us exclude hormones from formal consideration.

BAUER: When you say "polarization," do you mean spatial distribution.

HEANEY: No; I mean more than spatial distribution. When a cutting cone of osteoclasts begins to tunnel out a haversian cavity, the cone proceeds in a definite direction.

BAUER: Distributed in space, anyway; that is what you mean.

HEANEY: Osteoblasts are recruited from their progenitor cells to lay down bone at very specific places within the bone, at specific rates, and in specific quantities. Similarly osteoclasts are recruited; they resorb bone, and they do so at specific places and at specific rates. That they do this is an activity proper to bone simply because it is bone.

To be very certain that everybody knows exactly what I hope we shall talk about in this discussion, I would like to give an example from a completely different field, the rhythmic contractility of a tissue such as cardiac muscle.

We know that skeletal muscle in the resting stage is able to maintain a gradient of sodium and potassium across its cell membrane virtually indefinitely, whereas cardiac muscle has an intrinsically leaky membrane. The sodium ion tends to leak across the cell membrane until a critical gradient is reached; at this point depolarization is triggered, the cell is activated, and then during the recovery phase the sodium is pumped back out. This occurs spontaneously, and the period of this rhythm is determined, obviously, by the leakiness of the cell membrane.

The rate at which the heart works is determined by nerves, hormones, humoral agents, drugs, and one thing or another which act on this system; but the important point is the fact that all of these agents act upon this intrinsic rhythmic contractile system. Rather than directing our attention to the regulatory factors of a higher order of magnitude, such as the nerves and the hormones themselves that circulate through the blood and influence cardiac rate, let us direct our attention to the mechanism on which these factors play; that is, the intrinsic, rhythmic nature of the contractile system itself.

In bone we are not concerned with a rhythmic cell depolarization—at least I do not know that we are—but we are concerned with an equally intrinsic sort of tissue activity—the reconstruction of bone tissue as tissue. We have discussed the effects of a variety of recognized hormones on bone in the homeostasis of the extracellular fluid



calcium. Now, I would like to descend one whole order of magnitude to the tissue mechanism, not the biochemical mechanisms per se, but the tissue mechanisms on which these various hormonal factors play. I think this approach has a practical importance aside from the theoretic and biologic significance because these local factors exert very prominent effects on metabolic bone disease; perhaps we have not always looked far enough and wide enough to recognize their existence.

I first began to think seriously about this matter 10 years ago when working with Dr. Whedon at the National Institutes of Health. We came upon some unexpected results in an experiment. (See ref. 78.)

I will try to avoid going into a lot of detail about this experiment because the nature of the experimental system is not germane to the point which I want to make. Suffice it to say that we had calcium and nitrogen balances on a patient with active rheumatoid arthritis prior to and during treatment with prednisolone (fig. 42).

WHEDON: The patient also had osteoporosis.

HEANEY: Yes; that is in the record.

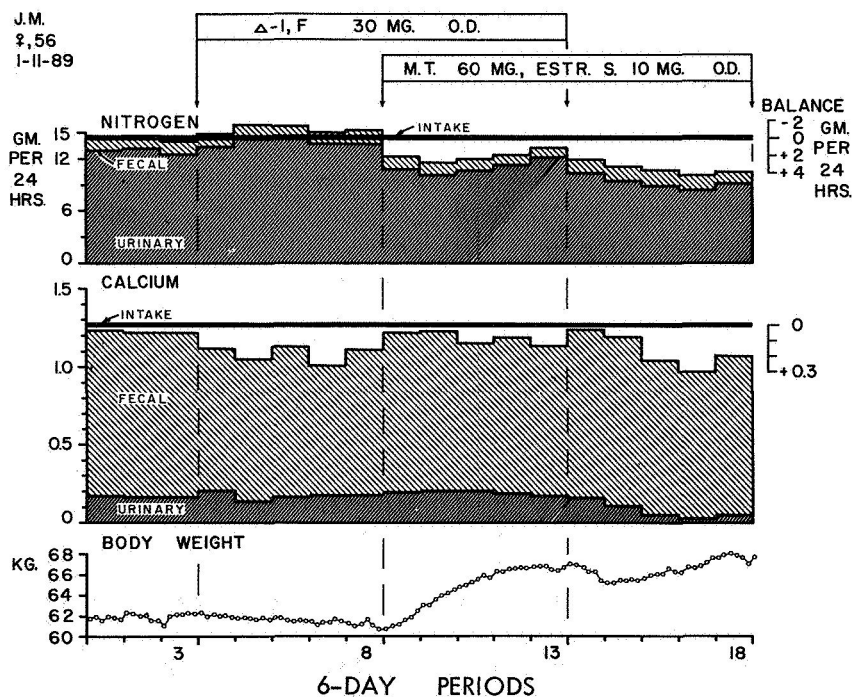


FIGURE 42. Nitrogen and calcium balances and changes in body weight in a 56-year-old woman with active rheumatoid arthritis and osteoporosis of the spine. [From ref. 78; reprinted by permission of the author.]

WHEDON: I mentioned that because it has everything to do with how it all comes out or does not come out, according to how you expect it should.

HEANEY: Again, I would rather not explain or attempt to explain the very complicated system here. Suffice it to say that during the control period this patient was in approximate nitrogen equilibrium with a very slight positive calcium balance on a high calcium intake. We had anticipated, perhaps naively, that when we put this patient on a high dose of corticosteroids, she would go into negative calcium balance. This is expected in normal people. It is certainly a feature in spontaneously occurring Cushing's disease and so forth. But instead of going into negative calcium balance, the patient had, in fact, increased her calcium storage during the period of medication.

This seemed paradoxical to us at the time and still does to me, although I have a number of explanations for it which I will avoid talking about at this time because they are not germane to the point of the discussion.

This observation has been repeated many times. It is not a uniform observation and cannot be found in all patients with rheumatoid arthritis or in rheumatoid arthritis with osteoporosis; but it does occur in many arthritic patients, and it has been reported by other people as well. It suggested to us that there may be some difference in the bone that is adjacent to active rheumatoid arthritic inflammation.

Calcium kinetic studies performed in patients of this sort have demonstrated the expected cortisol or corticosteroid effect; that is, a decrease in total body structural turnover of bone. This effect is difficult to explain in view of this positive balance shift. Certainly, clinically one expects the osteoporosis, if nothing else, to not get better and perhaps to become worse.

These observations prompted a number of other experiments. To make a long story short, we have been able to make rough measurements of bone accretion rates, as defined by Bauer et al. (ref. 79), in isolated segments of the intact limbs of patients with rheumatoid arthritis (fig. 43). The numbers along the abscissa represent successive 3-inch segments from the tip of the fingers on up the arm. They are numbered consecutively for convenience. The metacarpal-phalangeal region would be No. 2 and the wrist region, No. 3. The control is shown in solid black and prednisolone treatment, cross-hatched.

Even though in this patient, as a whole, the total body accretion rate fell as a result of corticosteroid treatment, there was no decrease in the bone mineral accretion rate in these segments during corticosteroid treatment. This experiment has been repeated many times and is a uniform finding.

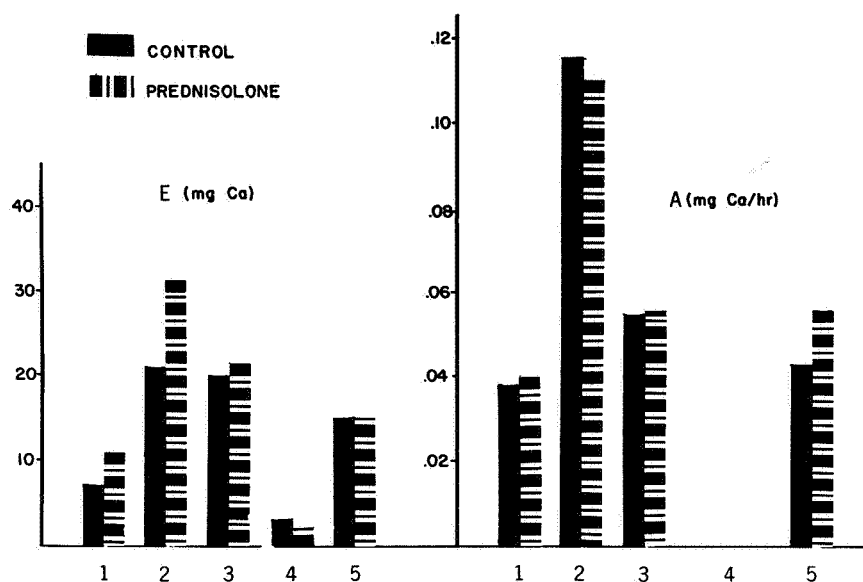


FIGURE 43. Bone accretion rates in patients with rheumatoid arthritis.

It is not apparent in this isolated figure, but the levels of accretion shown are from two to five times what one would find in normal people; thus there is increased structural turnover of bone around these inflamed joints, and this increased structural turnover is not depressed, at least over our periods of observation which are in weeks or months, by corticosteroid treatment, even though the sum total of all the bone in the patient's body did show a net decrease in bone mineral accretion. This emphasizes, I think, that there was a local factor in the bone adjacent to the inflammation, a local factor which was more important than, or which modified, the expected bone tissue response to a superimposed systemic or hormonal controlling agent.

URIST: Do these data mean that the positive accretion occurred in the area of the involved joints?

HEANEY: By positive accretion you mean positive calcium balance, increased bone mass?

URIST: Yes.

HEANEY: I did not measure this. It would be exceedingly difficult to measure, short of very sensitive densitometric methods which I have not employed. What I have measured is the bone accretion rate by these techniques, in localized segments.

URIST: Name the area included in each segment.

HEANEY: Around and including the involved arthritic joints, but the accretion is, I assume, going on in the bone rather than in the joints.

HOWELL: How old were these patients that you studied? In older rheumatoid arthritic patients, there may be considerable hypertrophic bony growth at the margins of the joints; this growth might affect mineral accretion in one direction. In adjacent subarticular bone, osteoporosis is a common early finding, and I should think that it is there that one might expect to register quite different mineral accretion rates.

HEANEY: These patients had osteoporosis, as observed by X-ray, but it does not matter for my purpose in this discussion that they did, because whatever it was they were doing, it was not depressed by an effective therapeutic dose of corticosteroid. This is the only point that I want to make. These local regions did not respond as the skeleton at large did in the same patient, nor did they respond in the way one has become accustomed to expect normal bone to respond.

NICHOLS: Do we know how normal bone responds to cortisone in a biochemical sense at the cellular level?

HEANEY: No. I am not talking about cellular level. I want to steer between the hormonal and the biochemical level and, somehow, end up at the tissue level itself.

LLOYD: Could you give us some more information as to how you calculated the "A" values for these particular cases?

HEANEY: I would like to but will resist the temptation because it would be a digression. I would be glad to do so at a later date when we have more time.

I am attempting to give examples of what I consider to be the effects of local factors. After I have given a few of these examples, I shall ask Dr. Talmage to give some further examples. We were interested in some of the physiologic mechanisms involved in the development of disuse osteoporosis. In one experiment designed to attack this problem, we cut all of the nerves in the lumbar plexus on one side of 200-gram young-adult rats; this caused marked muscle atrophy of one leg together with disuse osteoporosis on that side, rapidly developing over a period from 4 days to 3 weeks after nerve section. During this time we took measurements of the calcium content of the bones concerned and measurements of the uptake of radioactive calcium given at varying periods prior to sacrifice.

I will not go into any further detail about this aspect of the experiment because I do not want to talk about disuse osteoporosis. I do want to talk about some local factors and how they influenced our results—not to explain these factors but to show how they influenced our results.

We separated the bones into four regions. First, we took the femoral head and intertrochanteric region. We had to cut this off somewhat arbitrarily, but tried to be as precise as we could. Second,

we studied the remainder of the femoral shaft. Then we studied the knee, which consisted of the distal epiphysis of the femur and the proximal epiphysis of the tibia. These were readily separable because one could simply snap them off at the existing cartilage plates. These were almost fully grown rats so that the plates were not very active, but we could nevertheless separate them. Finally, we studied the remainder of the tibia-fibula complex.

Figure 44 is schematic and illustrates the changes in total calcium and in  $^{45}\text{Ca}$  uptake in these various regions. The top line deals with denervation alone. I will touch on the others in a moment, although they are not as important as the point I want to make here.

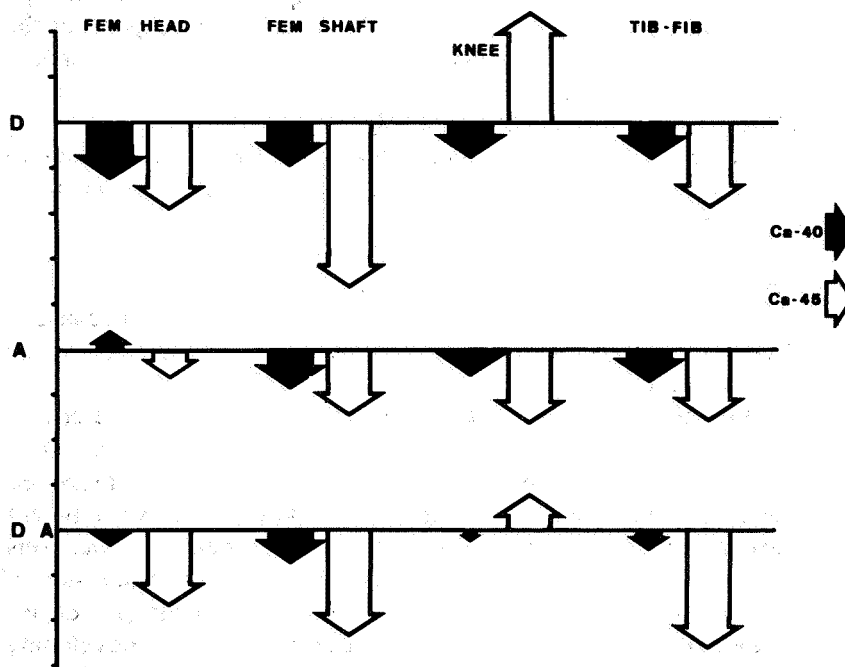


FIGURE 44. Changes in total calcium and in  $^{45}\text{Ca}$  uptake in femoral head, femoral shaft, knee, and tibia-fibula of rats after denervation (D), arterial ligation (A), or denervation and arterial ligation (DA).

Over the period of our study, which in this case was during the 10 days following nerve section, there was a fall of between 8 and 15 percent in the total calcium content of the bones concerned as compared with the content of control bones on the other side in the same animal.

But in each case, with one exception, the  $^{45}\text{Ca}$  uptake fell even more than did the total calcium content, so that a decrease in new bone formation may have contributed somewhat to the development of the osteoporosis. I will not go into this, but I want to point out that the

knee was the striking exception. Although these epiphyseal plates were fairly inactive they were, as we all know, not fused; the primary spongiosa, the zone of provisional calcification and so forth, were on the shaft side of where we split them, so that all one had here were the epiphyses themselves. Consistently over 2 years in about 30 groups of 5 rats each, we very reproducibly found this considerable elevation in uptake of radioactive calcium by the bone in the knee area, which is quite different from the effect through the rest of the leg. The knee, too, developed disuse osteoporosis at perhaps a slightly slower rate than the other area, but it did so at an elevated rate of new bone formation, whereas the others did so at a depressed rate of bone formation.

MACDONALD: What was the time interval between isotope administration and denervation?

HEANEY: We studied various permutations and combinations with this. We did some 3 or 4 days after denervation and others as long as 10 to 12 days after denervation. Then we sacrificed animals at various intervals from 1 hour to 30 days after isotope injection. This is only one representative schematization of the type of result. Usually, the ratio of the isotope uptake of the operative side versus the normal side was constant throughout the period of study.

I would rather not go into too much detail about how you get disuse osteoporosis, which is another topic entirely. I cite this experiment simply as an example to show that the response of the bone of the epiphyses to the hormonal and mechanical requirement of that leg was different from the response of bone above and the bone below it.

NICHOLS: This was epiphysis per se and not metaphysis?

HEANEY: This was entirely epiphysis. If you attempt to snap these epiphyses off, they come off quite readily and quite reproducibly, and the active zone of cartilage calcification goes into the shaft side, so it was nothing but isolated epiphysis.

RAISZ: Denervation is not the same as disuse. I wonder if the bones were hypertrophic in part because of trauma to the denervated joint.

HEANEY: They undoubtedly did undergo some trauma, but it is difficult to say exactly how much or what effect this had.

COPP: I would like to comment on this point. Shim et al. (ref. 80) also denervated the hind limb in rabbits and observed an increase in bone blood flow in the foot, although there was no significant change in bone mass.

HEANEY: The second section of our experiment was to do the same thing with as complete a ligation of the arterial supply to that same leg as we could get. In this case it was done with arterial ligation alone. There is obviously room for collateral circulation, and this plainly did occur. We controlled this circulation by some postmortem injections of Micropaque into the aorta and took X-rays so that we

could see what the vascular pattern looked like. There was blood flow into the limb, but it came in through collaterals; we could see retrograde flow back up the femoral arteries to the point of ligation, and the ligations remained intact.

There was always a great deal more barium on the intact side than on the ligated side, but there was blood flow there.

With decreased blood supply there was decreased  $^{45}\text{Ca}$  uptake in all segments, again repeated at varying intervals. Curiously, the femoral head region gained bone and became, if you will, slightly sclerotic, whereas the other regions became somewhat osteoporotic. When we combined arterial ligations with denervation we obtained the results shown at the bottom of figure 44. The knee region again showed increased radiocalcium uptake despite decreased overall blood flow and almost no change in the bone mass itself.

HOWELL: By those nerve ligations you released tension communicated to the epiphysis as muscle tone. Did you also study the effect of cutting ligamentous insertions about the knee, inasmuch as these would retain a mechanical stress at points immediately adjacent on the epiphysis?

HEANEY: We did not in these animals. We performed another experiment in rabbits by cutting the heel cord, which may or may not be what you are getting at.

HOWELL: I thought perhaps the stress remained applicable to the epiphysis because of the attached ligaments.

HEANEY: These bones also became atrophic, Dr. Howell. They lost at essentially the same rate as the shafts above and below them. Ultimately, if you carry the experiment out long enough, bone loss increases to 20, 30, or 40 percent.

Again, I only want to point out that one geographic region of this animal's leg bone responded differently from another geographic region; therefore, there are many factors which may be invoked that one can think of to explain differences. It is precisely some of these factors that we will be discussing, so I would rather not beg the question.

Dr. Talmage, you have some observations which might fit in now with respect to differences in local bone responses to systemic stimuli.

TALMAGE: It is obvious that everything in our figures will point to effects of parathyroid hormone. However, in line with Dr. Heaney's request, we will temporarily ignore the parathyroid implication and concentrate on the differences in response of two different parts of rat bone, the metaphysis and the shaft of the femur, in regard to the various parameters studied. For our studies, the metaphysis begins at the epiphyseal line moving proximally. The shaft is composed almost entirely of compact bone.

NICHOLS: Is the epiphysis included?

TALMAGE: Only the epiphyseal line—the cartilage is cut off as cleanly as possible. We are trying to deal only with bone; we feel we have succeeded. I would like to reemphasize the fact that there is a very marked difference in the level of equilibration when metaphyseal bone and diaphyseal bone are incubated in serum. Figure 45 is a graph which shows that whether one starts with low or high calcium in the incubating medium, metaphyseal bone produces a higher calcium level in the medium than does diaphyseal bone. This difference can be further exaggerated by incubating the same portions of the femur taken from animals nephrectomized 24 hours before use. In bone from such animals, the equilibration level for the metaphysis is at an even higher calcium concentration, while that for the diaphysis is essentially the same. With normal animals, the phosphate levels in the medium after 4 hours of incubation follow those of calcium.

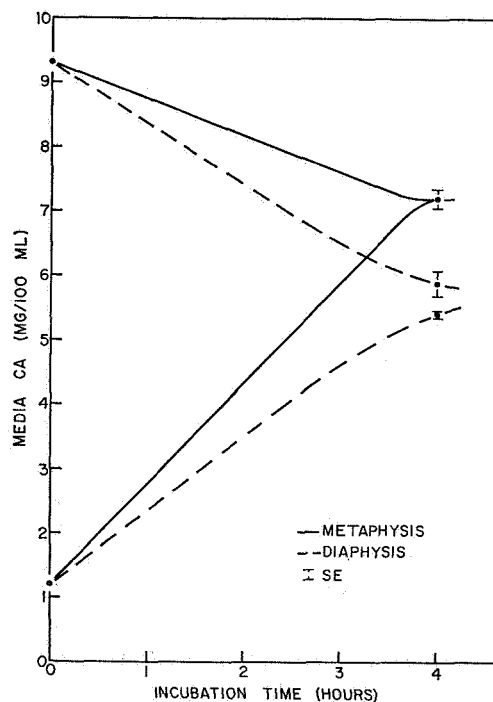


FIGURE 45. Incubation of metaphyseal versus diaphyseal bone in media with normal and low initial calcium levels. Metaphysis differs from diaphysis by  $p < 0.005$  at hour 4. [From Cooper et al., ref. 81; reprinted by permission of the publisher.]



These are also higher in the serum after metaphyseal equilibration if bones from nephrectomized animals are used. Since both calcium and phosphate are raised, this indicates a change in the solubility of the calcium phosphate complexes in the metaphysis due to nephrectomy.

PRITCHARD: Is this live bone or dead bone?

TALMAGE: These are live bone incubations; however, the results utilizing dead bone are the same, except that the 4-hour levels are slightly lower in all cases (ref. 81).

The difference in the production of organic acid by these same two areas of bone is given in table IV. Per milligram of bone collagen, there is much more production of citric acid in the diaphysis than in the metaphysis at the end of the bone-serum incubation. Remember, this is per milligram of collagen. Since there are less cells per milligram of collagen in the diaphysis, the differences between the two areas of bone would be greater should the results be based on citric acid produced per cell.

TABLE IV  
INCUBATION OF RAT FEMUR IN SERUM<sup>a</sup>

	Metaphysis	Shaft
Citric acid production, mg produced/g bone collagen.....	1.3	<sup>b</sup> 3.0
<sup>45</sup> Ca removal, following 2-week incorporation, cpm/ml serum...	523	<sup>b</sup> 400

<sup>a</sup> Incubation period was 4 hours.

<sup>b</sup> Values after incubation of bones from parathyroidectomized rats in serum from control rats were significantly less than that for bone taken from parathyroid-intact animals.

A comparison of the amount of radiocalcium removed from these two areas of bone during incubation is also listed in table IV. In this particular experiment, the radiocalcium had been in the animal for 2 weeks prior to sacrifice. There is still more radioactivity removed from the metaphysis than from diaphyseal bone. However, if I may bring in the parathyroid hormone again briefly, it is in the diaphysis and not in the metaphysis that the effects of the hormone can be seen in regard to radiocalcium removal.

Figure 46 shows differences in the effect of the hormone on the rate of incorporation of <sup>3</sup>H-cytidine into RNA extracted from metaphyseal and diaphyseal bone after incubation in serum. After 20 minutes of stimulation by a calcium-free peritoneal lavage, a procedure for increasing endogenous parathyroid secretion, there are opposite effects in the two types of bone. There is an increase in uptake in the me-

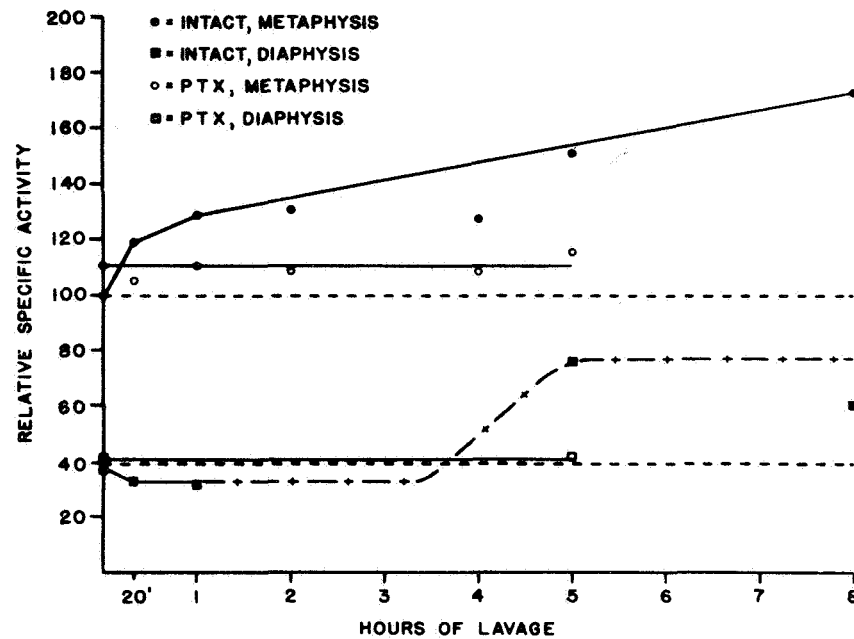


FIGURE 46. Incorporation of  $^3\text{H}$ -cytidine into RNA. Control metaphysis is expressed as 100.

taphysis and a decrease in the diaphysis. After 4 or more hours of continuous stimulation, the incorporation of  $^3\text{H}$ -cytidine is increased in both types of bone. I will refer to these figures again in the discussion of cell modulation. They are introduced here to illustrate another difference between these two areas of the femur in the rat.

Figure 47 illustrates the uptake of  $^3\text{H}$ -thymidine into DNA. The primary difference between the two areas of bone is limited to the greater response in metaphyseal bone.

In summary, then, there are major metabolic differences between metaphyseal and diaphyseal bone, as might be expected. However, they also respond quite differently to stimulation by parathyroid hormone; in the case of incorporation of  $^3\text{H}$ -cytidine into RNA, for at least a limited time, opposite effects were produced. Obviously, therefore, it is important to ascertain just what type of bone is being studied when using endogenous or exogenous parathyroid hormone, since opposite effects may occur in different parts of bone. This may be just as true in regard to other hormones.

HOLTZER: How many nonbone cells are involved in this?

TALMAGE: How many nonbone cells?

HOLTZER: Out of the total population—marrow cells.

TALMAGE: Marrow cells, leukocytes, and the like? In our incuba-

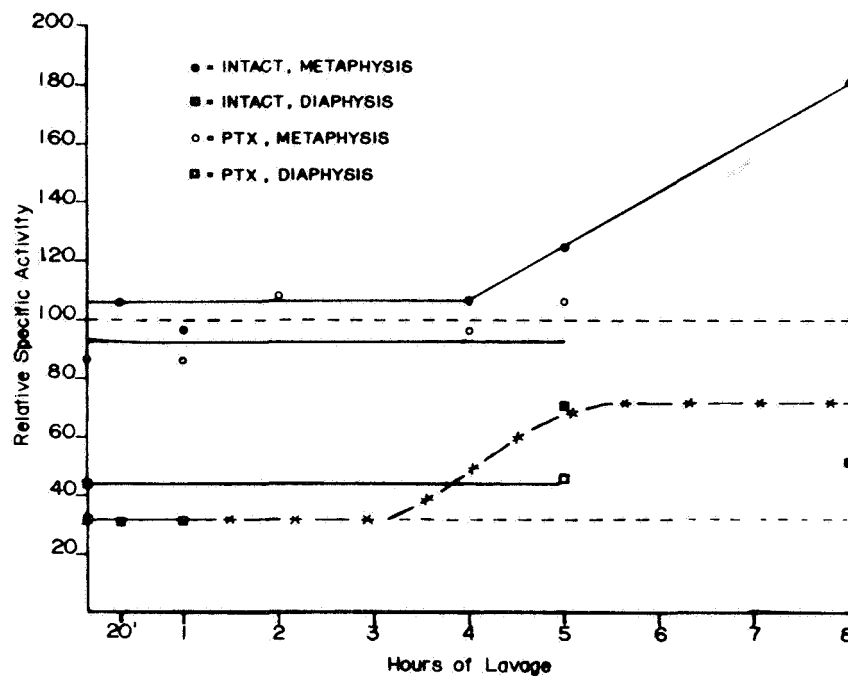


FIGURE 47. Incorporation of  $^3\text{H}$ -thymidine into DNA. Control metaphysis is expressed as 100.

tion procedures, we wash out the marrow cells before the bone chips are incubated. This washing procedure must be very gentle, as the osteoclasts will not stand much stress and are easily destroyed. We believe we get rid of most of the marrow cells, leaving some leukocytes and those red cells located in the sinuses of the metaphyseal bone.

HOLTZER: There is a large number of vascular cells, tissue cells?

TALMAGE: Yes.

HOLTZER: So this kind of measurement, while certainly suggestive, would be more or less difficult to interpret, would it not, just on the basis of the response of the osteocytes and osteoblasts in the system?

TALMAGE: I did not want to include the osteocytes in this—

HOLTZER: But you are talking about bone—although, in fact, you have a lot of nonbone cells in the system.

TALMAGE: Obviously we have nonbone cells left in this system; there are probably more in the metaphysis than in the diaphysis, because many of these cells are trapped in trabecular bone. However, we are studying parathyroid effects. As yet, this hormone has not been accused of affecting blood cells; so we cannot see how the nonbone cells can be important in our discussion.

RAISZ: The physical difference in these two kinds of bone is very

striking, and it seems to me for that reason that the preparation method is going to affect the way the cells come out. Would you give us more details on how the material is obtained for the biochemical studies that you do?

HEANEY: Are you referring to the cytidine and thymidine studies, or the prior studies?

RAISZ: The citrate and the thymidine studies.

TALMAGE: When the animal is killed, the femur is removed and broken at the epiphyseal line. The cartilage is cut off from the femur with a razor blade. The bone is then cut to separate the metaphysis from the diaphysis. Each portion is gently washed out twice with saline from an eye dropper. Each section is broken into several fragments and placed in the incubation fluid. The total time taken for the entire procedure is 8 minutes.

RAISZ: The diaphysis is simply cut up into fragments?

TALMAGE: And also the metaphysis.

RAISZ: There is a large number of cells inside the diaphysis whose access to the medium is fairly distant.

TALMAGE: That is right.

RAISZ: Whereas there is not such a large number in the metaphysis. Is that correct?

TALMAGE: In distance, yes. But I do not feel that the fact that the osteocyte is away from the surface inhibits it from receiving nutrient. I think there is a very rapid transport into the osteocyte.

NICHOLS: Dr. Owen and I can support that. Within a 4-hour incubation, Dr. Owen, by autoradiography, was able to show proline within the osteocytes in fragments of bone incubated *in vitro* with radioproline in a fashion similar to Dr. Talmage's.

RAISZ: You are studying rates, and as long as you are studying rates, the differences here must be pertinent. I agree that things get in and out, but I cannot believe that there will not be a difference between the rate with which they get in and out through lacunar channels of various sorts, and the rate directly from the medium to the cell. I think these rates are critical to the way in which your system is going to behave metabolically.

NICHOLS: No one will disagree with you about that. As far as I know, however, no one has done a careful study to see how soon the label appears in the surface cells compared with those in the depths, unless you have, Dr. Owen?

OWEN: No; not in tissue culture.

TALMAGE: In tissue culture of embryonic mouse radii, every cell had tritiated glycine in it after one-half hour of incubation. Of course, the distance here was only a few microns.

RAISZ: That is why we use embryonic tissue. We cannot get

a late, highly mineralized embryo to do the things we can get early embryonic bone to do. We have assumed that this was because highly mineralized tissue was not well nourished in the absence of a pulsating circulation which increased both perfusion and mixing.

TALMAGE: I would like to point out that in the incubation procedure there may be less contact with osteocytes than with surface cells. If this is true, then it would even further exaggerate the differences demonstrated between the diaphysis and the metaphysis.

RAISZ: I do not know in which direction we are going.

TALMAGE: The differences are there already, and if we increased the ease of communication with the osteocytes we simply further exaggerate these differences.

PECK: I think the differences that you pointed out are fascinating. I think it would be equally interesting to know what the pool sizes of stable precursors were in the bones at the start of incubation; for example, the pool of stable cytidine with which the radioactive labeled cytidine would mix. You could explain the differences in incorporation between the two bones merely on the basis of isotope dilution if there were large differences in pool sizes of stable material. Do you have any information about this?

TALMAGE: No; but I would agree that if there were large differences in pool size you would have dilution effects. However, could this not still be a transport problem?

PECK: Particularly, as has been suggested, if there were different cell types.

PRITCHARD: Is the cytidine going to new RNA or exchanging with old RNA?

TALMAGE: Unless my information is wrong, the cytidine goes first into a new messenger RNA.

HOLTZER: That does not follow. It is not true that all of the rapidly incorporated cytidine and uridine must go into messenger RNA. Any autoradiographic study, whether with an ameba or with bone cell, suggests incorporation first into the nucleolus and later into the cytoplasm. There is now much evidence which suggests that the nucleolus is responsible for synthesizing ribosomal RNA. Where and when messenger RNA is made in tissue cells is by no means clear.

But I agree that uridine and proline will penetrate into tissues several millimeters in thickness. In less than 5 minutes these isotopes will penetrate into a piece of cartilage over 2 millimeters in thickness (ref. 82). But I would emphasize, again, that it would be interesting to know exactly which cells are responsible for all the activity you are describing.

TALMAGE: Naturally we are attempting to do that. We have separated out a fairly pure preparation of osteoclasts. Also, we

have a mixed preparation of mesenchyme cells and osteoblasts, and we hope very shortly to have a pure preparation of osteocytes. We are working on it.

RAISZ: I think it should be pointed out that embryonic cartilage is quite different from bone with respect to diffusion between cells.

HOLTZER: Is there any evidence for that? How can you—

RAISZ: Polarize tissue?

HOLTZER: Yes; but is there anything more charged than, say, chondroitin sulfate in terms of exclusion? I would not have thought that only on the basis of its charge, cartilage would be more difficult to penetrate than bone in terms of an ion-exchange kind of effect.

RAISZ: But not in terms of water content.

ARNAUD: I would like to know whether or not any of the studies done up to this point have been carried out in grossly marrow-contaminated bone and whether or not the results were different.

NICHOLS: Can I speak on this point, because I think it is a critical one? I will come back to it again when I show you metabolic data about marrow and bone cells derived from the same bone sample.

The whole problem of the importance of marrow contamination came up some time ago. It turned out that the percentage of the total cells which are demonstrably marrow is not terribly large, but there is no good way of measuring this precisely; the metabolic patterns of the marrow cells are somewhat different, and the total metabolic activity tends to be less than that which we see in the bone. Therefore, the fraction of the metabolic activity that can be attributed to contamination is probably quite small, 10 percent or so.

With respect to the problem of the different pool sizes into which RNA precursors are assimilated, we have some data that relate to the cellularity of the tissue and therefore indirectly to pool size. If one plots the DNA or RNA content per weight of different kinds of bone against age in months of rats, one gets curves like those in figure 48. The age groups range from 3 weeks (the minimum age at which I felt I could separate metaphyseal, trabecular, and cortical bone clearly) to 22 months (the age at which half the animals had died). The curves for DNA show clearly that the cellularity of the two types of bone is quite different—cortical bone being, of course, always the lesser of the two. More important, however, is that while the cell content of cortical bone decreases steadily with age, the cell content of trabecular bone actually rises up to the age of 4 months and then falls abruptly.

Although the RNA data only extend to 8-month-old rats, they follow similar patterns. Again, the RNA content of cortical bone falls rapidly with advancing age up to 4 months (the point where growth has leveled off), and then declines rather slowly. RNA in trabecular bone starts lower than in the cortex but remains stable through the

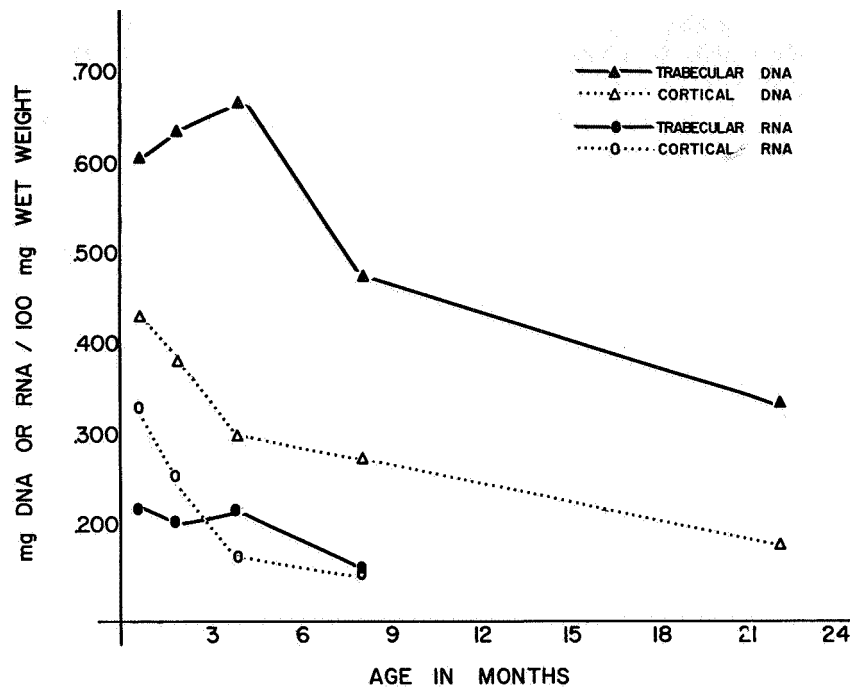


FIGURE 48. Content of DNA and RNA per weight of trabecular and cortical bone of rats.

growth period only decreasing after 4 months of age. These data show that there is indeed a considerable difference in pool size with differences in age and in bone type. Therefore, unless the pool size is known, your observations—while interesting and provocative—cannot really be interpreted.

HOLTZER: Are you calling cells the pool size?

NICHOLS: Yes; in the sense that the pools are in the cells, and hence the overall size of one is related at least indirectly to the size of the other.

I have done one other experiment regarding cells and their activities that might be of interest in this discussion. This experiment was performed only once, and I am not sure how quantitative it is. However, if one takes chips of bone and separates the cells from them by grinding in a mortar with successive batches of medium—the way we do (each batch is poured off, the fragments of calcified collagen are allowed to settle for 30 minutes, and the cells are finally harvested from the resulting supernatant by centrifugation)—one obtains a progressively smaller number of cells with each successive grinding, until all the fragments have been ground away.

Thinking about this procedure, it occurred to me that as one grinds, the first cells to come off are probably mostly marrow. The next are probably surface cells, while the later batches contain larger and larger proportions of osteocytes, until the final ones should contain virtually nothing else.

It turned out that when the cells in each of the five successive grinds were examined, there was a progressive difference in their metabolic patterns. Very simply, it seemed that as the osteocyte is approached, the oxygen consumption per milligram of cell DNA goes down to virtually zero, and the lactate production, very reasonably, goes up. Moreover, looking at a smear of the cells under a light microscope, one finds that the first grinds contain mostly round mononuclear cells with moderate amounts of cytoplasm, while the last ones are elongated with scanty cytoplasm and a nucleus which seems to bulge the cells in the middle. Now, whether these are really osteocytes, I do not know, and I do not know what their electron micrographs look like; I only say I think they might be osteocytes.

HOWELL: Is that from cortical bone?

NICHOLS: Yes; from rats about 50 days old.

PECK: One important point about the problem of working with whole tissues and individual cells *in vitro* is the possibility of leakage of cytoplasmic enzymes (ref. 83). Some tissues, in which cells are encased, may provide greater protection against loss of enzymes than other tissues, in which the cells are widely exposed to the bathing medium; compare, for example, diaphysis with metaphysis or whole bone segments with isolated cells.

Dr. Talmage, it would be interesting to study the relative appearance of enzymes in the medium during *in vitro* incubation of metaphyseal and diaphyseal bone; for instance, those enzymes associated with citrate metabolism.

PRITCHARD: When bone is grown *in vitro*, the cells walk out one by one. The first cells that emerge do not have much phosphatase in them, but the second wave shows normal phosphatase activity. Could you not get a useful separation of cells just by letting them wander out under their own steam *in vitro*?

NICHOLS: There you get into the problem, do you not, of possible dedifferentiation as the walk starts.

PRITCHARD: Yes, I suppose so; but it is worth a try.

HEANEY: From listening to this discussion I have the impression that some of the differences Dr. Talmage has cited might be a result of artifacts in the study method rather than a result of the local factors with which I am concerned; therefore, I would like to return to our topic lest it evaporate entirely.

A number of years ago, Dr. McLean and his collaborators published



some experiments on the effect of parathyroidectomy on haversian bone remodeling (ref. 84). I know that he has done some additional work in this field since this publication and that this work is in keeping with our topic of excluding hormones, rather than including them.

MCLEAN: Would you like me to introduce the subject of internal remodeling?

HEANEY: Please.

MCLEAN: I am not equipped to give a didactic lecture at this time, but I have some figures to present. I would like to recapitulate, particularly for the benefit of those who are not thoroughly familiar with this subject, what goes on in internal remodeling.

In 1853, Tomes and De Morgan (ref. 85) described what they called absorption in bones. They showed that the cavities assume the form of tunnels, of which figure 49 is a cross section taken from Petersen (ref. 86); that when absorption was going on, these tunnels were lined



FIGURE 49. Ground section of human bone showing formation of osteons. During absorption, tunnel lined with osteoclasts, *a*; reversal of process during the filling in of the tunnel by osteoblasts, *b*; tunnel filled in by the deposition of successive lamellae, *c*, with a haversian canal. [From ref. 86; reprinted by permission of the publisher.]

with osteoclasts; and that when they reached a certain size, fairly uniform for an individual, the whole process was reversed, and instead of osteoclasts, the tunnel became lined with osteoblasts.

Tomes and De Morgan made a very astute remark about this. They said that as far as they knew, the same cells that accompanied the resorption of bone were not building new bone. I think we still believe that these are the same cells, which reverse their functions, change their form, and start making new bone.

HEANEY: There is some disagreement with that comment, Dr. McLean; shall we postpone that discussion for now?

MCLEAN: Yes. To continue, the tunnel is now lined with osteoblasts, and it is filled in by the deposition of successive lamellae, everything moving toward the center, so that eventually we come down to the haversian canal that was shown previously in Dr. Robinson's material.

Dr. Robinson also showed a narrow band of uncalcified tissue; he referred to it as prebone. I prefer to use the term "preosseous tissue," or it may be called osteoid. There is a very thin layer of uncalcified matrix, mainly collagen and ground substance; immediately around this layer is the calcification front (shown in previous electron micrographs), the little rough areas where new mineralization takes place.

We have then, in a working osteon, several morphologic landmarks. First, there is the lumen of the osteon, which contains the blood vessels and whatever else is carried within the haversian canal; around the lumen is a thin layer of uncalcified tissue; then there is a layer of very reactive tissue. I will show in a moment what goes on in the reactive tissue. Everything beyond the thin layer is calcified. This area calcifies very quickly, up to something like 70 percent of its final mineral content within a matter of days; it may take weeks to add the other 30 percent.

The particular point that I want to emphasize is that the reactivity in this osteon is almost entirely limited to the calcification front. The uncalcified osteoid zone is not reactive, except that it will take up sulfated mucopolysaccharides. They are part of this structure and they are incorporated within this layer. But this layer is not yet calcifiable; suddenly it becomes calcifiable and the calcification process moves in.

Concentric lamellae are formed until the canal finally narrows down to its ultimate dimension, averaging approximately 20 microns in diameter, and then all new growth ceases. There is no longer a zone of preosseous tissue; there is no more mineralization. Mineralization has been completed, and the entire structure then becomes a resting osteon.

Some people disagree with this characterization, but Jacques Vincent

introduced the terms "metabolic" and "structural" bone. In his terms, the metabolic bone is at the margin of the calcified area, and I think he had some idea, also, that the partially calcified osteon is reactive until mineralization is complete. This work was subsequently published by Vincent and Haumont in 1960 (ref. 87).

This sequence is illustrated in table V (ref. 88) which lists five zones. Zone A is the haversian canal lined with osteoblasts. It can be stained with histologic stains, but there is no particular reactivity in the membrane of the canal, and there is nothing different in these osteoblasts from those in other parts of the skeleton. Zone B is the preosseous tissue, which Frost and Villanueva (ref. 89) have called the osteoid seam. This can be seen with histologic stains, and it takes up radiosulfate *in vivo*; so that if one gives  $^{35}\text{S}$  to an animal, some of it will be deposited in this zone.

Zone C contains all the activity. This calcification line (calcification front or calcification zone) will take up  $^{45}\text{Ca}$ , radium, strontium, phosphorus, cobalt, lead, tetracycline, and alizarin, all in the same place. This is also the zone where zinc is deposited in the normal metabolism of the animal. If radioactive zinc is given, it is also deposited in this zone. Cadmium competes *in vivo* with zinc; both appear to react as trace metals which catalyze some reaction and possibly involve catalysis of mineralization. This zone takes up Sudan Black

TABLE V  
CROSS SECTION OF FORMING OSTEON, FROM CENTER TO PERIPHERY

Zone	Description	Reacts with—
A.....	Haversian canal, lined with osteoblasts and housing blood vessels, nerves, lymphatics, and connective tissue	Histologic stains
B.....	Uncalcified preosseous tissue (osteoid seam)	Histologic stains, $^{35}\text{S}$ <i>in vivo</i>
C.....	Calcification line (calcification front)	Mineral component, reacts with $^{45}\text{Ca}$ , $^{226}\text{Ra}$ , $^{90}\text{Sr}$ , $^{32}\text{P}$ , Co, Pb, tetracycline, alizarin, etc. Organic component, reacts with $^{65}\text{Zn}$ <i>in vivo</i> (Zn also demonstrable in untreated animal), Cd (competes <i>in vivo</i> with Zn), Co, Sudan Black, and other stains
D.....	Partially calcified osteon.....	$^{45}\text{Ca}$ (after <i>in vivo</i> or <i>in vitro</i> pre-treatment with acid)
E.....	Cement line (external limit of osteon)...	Histologic stains

very specifically, and some other stains also. Therefore, this is the reactive portion of the osteon and accounts for nearly all of the reactivity.

Zone D is the partially calcified zone, and zone E is the cement line, the external limit of the osteon.

Figure 50 is a beautiful illustration from Leblond and Lacroix (ref. 90). Section A of the figure is the histologic section; you can see the calcification zone stained with cobalt. This zone also takes up Sudan Black. Next to it is the uncalcified zone, which is not reactive and does not stain with either cobalt or Sudan Black.

Section B is the microradiograph with arrows to localize the zones so that one can compare the histologic section with the microradiograph. Note that the preosseous tissue does not stain and casts no shadow in the microradiograph.

My particular point is that there are two things that give life to bone, and bone—far from being an inert structure with purely structural functions—has a great deal of life in it. One thing that gives life to bone is the osteocyte; it will be discussed further so I will not dwell on it.

I would like to go on for a moment with the idea that internal remodeling, which means the formation of new osteons, goes on throughout life. We used to say it went on throughout life but at a diminished rate with increasing age. Now we have decided that, if anything, remodeling is more active as the individual ages.

The reactive zone in the osteon forms a continuum between the fluid containing calcium and the calcium in the solid form, so that there is a line of demarcation, which was shown clearly in the electron micrographs; this line of demarcation constitutes the bridge between the fluid-state and the solid-state mineral. Whether this bridge is to be regarded as a solution of calcium already in combination with phosphorus or not is not clear; but it is evident that this line is the border zone, and it does afford a bridge from the fluid state of the solution of the mineral in the blood and the tissue fluid to the solid state in the bone.

It is my belief that this process is essential to life and that what we have been describing is essential to homeostasis and to the maintenance of a constant calcium concentration. It should be noted that we have described a one-way process; i.e., the mineral going into the bone. That is very easy to demonstrate. Whether mineral can also come out of this complex into the blood is something that still remains to be shown. I do not think we need to postulate that this mechanism has anything to do with the passage of mineral back into the blood. We have two excellent mechanisms for that, one with the osteoclasts and one with the osteocytes, so that the needs of the body for mineral

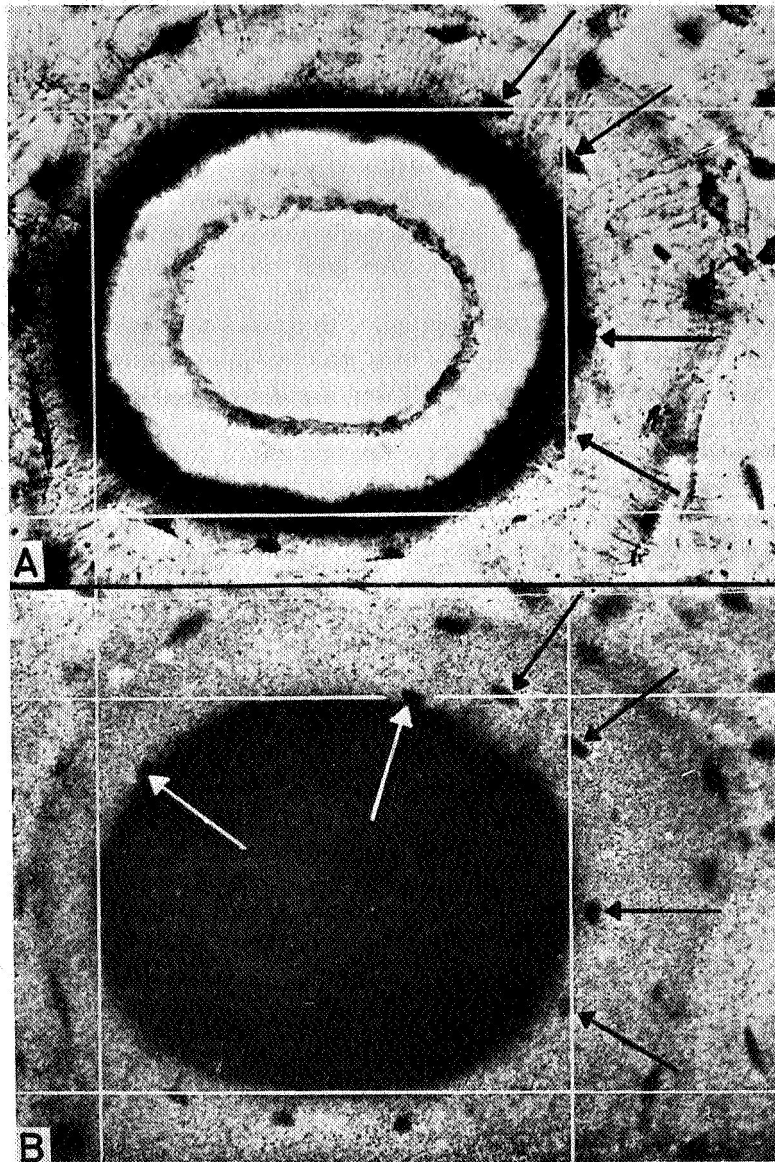


FIGURE 50. (a) Undecalcified ground section of compact bone treated with cobalt nitrate. (b) Microradiograph of the same section. Arrows indicate zones. 533 $\times$ . (See table V.) [From ref. 90; reprinted by permission of the publisher.]

to be returned to the circulation are taken care of by cells other than those in the osteon. But it is still true that the osteon is important

in the overall balance between mineral deposition and demineralization or resorption.

Several years ago with Jenifer Jowsey, we did some experiments on dogs to determine whether this resorption process in bone is under the control of the parathyroids (ref. 84). That question is still, I think, unanswered, but to my mind, at least, the weight of the evidence so far is that this process is not under parathyroid control. It goes on even in the absence of the parathyroid glands just as growth goes on. The remodeling incident to growth also goes on in the absence of the parathyroid glands.

Now, there is one more question. What triggers this mechanism? Amprino (ref. 91) gave me the idea that the particular locations, the areas in which new osteons will form, are determined by the stresses and strains on the bones. The amount and location of bone tissue undergoing resorption seem to be controlled by mechanical conditions characteristic for each skeletal location. Once the areas of skeletal tissue to be remodeled are determined by mechanical factors, then the actual location of the individual tunnels is apparently a random process. No one has offered any explanation as to what starts one of these tunnels forming or as to what determines a particular location for any of these tunnels. However, bone remodeling, whose first phase is resorption, comes into play when liberation of mineral from bone is not sufficient to meet the physiologic demand.

HEANEY: I think one could disagree with that because, although the specific localization might be random, the polarization is such that it could not be random, could it?

MCLEAN: I think it is true that tunnels never go across the bones. In the long bones they go in the long axis of the bone, but not precisely. As you said a while ago, they have a slight spiral turn.

URIST: Do you refer to the work of Amprino on stresses and strains after an experimental fracture?

MCLEAN: No.

URIST: How did he do that experiment? Has it been published?

MCLEAN: Amprino (refs. 91 and 92) himself referred to his postulates as assumptions, and stated that further research is needed to support them.

URIST: It is easy to demonstrate the bone deposit on the concave side as a response altered stress after a fracture, but it is more difficult to demonstrate the osteogenic response to stress on an unbroken bone. I would like to see someone put stress on an unbroken bone with some kind of apparatus and show that the resorption cavities begin to form chiefly on the side of the strain.

This also brings up another question. Let us assume that one makes cross sections of the bone and that where the bone is bowed, on the

convex side, there are more resorption cavities than on the concave side. Let us assume that one makes a series of cross sections, maps this out, and makes careful counts of resorption cavities. The question is, Is the difference the cause or the result of the bowing?

HEANEY: It is impossible, offhand, to answer that question; it is easier to ask it than to give an answer. I might cite an observation reported by Putschar (ref. 93) and quoted by Lent Johnson (ref. 94) in his encyclopedic chapter in the Henry Ford Hospital Symposium. Putschar observed a young adult who had practically complete poliomyelitic paralysis of both legs acquired at 1 year of age and showed subnormal osteonic remodeling; he stated that this certainly suggests that mechanical, in addition to genetic and metabolic, factors play an important part in the formation of cortical osteons.

PRITCHARD: I do not think anyone has much information on this point, but it is known that remodeling is very intense at the attachment of very powerful tendons, for example, at the linea aspera of the femur.

Then, of course, there is the work on the remodeling of vertebrae in scoliosis, and on the movement of tooth sockets with orthodontic appliances, in which a close linkup has been established between the known stresses and strains and the remodeling reactions of the skeleton.

HEANEY: I think we can say, as Dr. McLean has pointed out, that internal remodeling has considerable homeostatic significance, that is, significance for the extracellular fluid calcium homeostasis; but aside from that, remodeling will exist anyhow and is probably independent of parathyroid hormone. Certainly, in its topography it is independent of hormones involving calcium homeostasis. The real question we must ask is, Why is it here? What is it that determines that it is in this place, and that it is at all?

It has taken a while to get around to asking some of the fundamental questions, but I think that we must give some thought to the following questions. Does the aging of the bone material itself somehow exert an influence on subsequent turnover? That is, when bone is formed, does a cycle of absorption get initiated which ultimately predisposes to subsequent removal of the bone itself? What is the relationship between the nutrition of the bone cells and their blood supply? What role, if any, does this relationship play in subsequent removal and reconstruction? Finally, there is the obvious question, which we have all known about for a number of years, concerning the role of the mechanical forces and the electrical effects thereof. Are these mechanical forces, when all is considered, adequate to explain all that we know about and all that must be explained with respect to local factors?

We have not given formal consideration to these questions as yet, but it seems to me that these are some of perhaps many more which could be introduced. It is against this background that I would like

to have Dr. Currey talk, for he has given much thought to such questions. I would like to turn the discussion over to him now.

CURREY: In this gathering I may seem a bit of a heretic because I am not going to talk about mineral metabolism. I am going to talk first about haversian systems, or rather, internal remodeling.

Concerning haversian systems, there are two questions. Why do they form at all? Why do they form in the places they do? In fact, these two questions always get congealed.

People who have thought about haversian systems have produced about four reasons as to why they should be formed. One reason is that bone becomes necrotic; cells die and the bone is replaced by new, healthy bone. Another reason is that the haversian systems in some way make the bone mechanically a better job than it was before the haversian systems were formed. This was thought to be the case by reasoning from trabeculae. Trabeculae look like haversian systems out in space and are clearly mechanically adaptive. There are haversian systems in bone, and there are trabeculae in bone; both are mechanically a good thing.

Another suggestion was that haversian systems remodel the initial, rather rapidly put down, blood supply and make it more efficient. A final reason is that these systems remodel for mineral metabolism purposes, making new bone when calcium and phosphorus are available and so on, which we have heard about.

I would like to start by discussing the evidence that the exact place in which haversian systems are formed is determined by cell death. If you take a thin section out of bone (preferably cut by saw), fix it, grind it down but do not decalcify, and then stain, you get a very different idea of what bone looks like than if you decalcify it and stain the collagen and soft parts. There is a distinct difference between those parts of the bone where there are no cells, the lacunae are empty, and there are no nuclei showing, and other places where the nuclei stand out strongly.

If you take a young calf and look at its bones, every lacuna is filled with a nice, strong staining nucleus. If you take a man of 75, outside the haversian systems you find a great proportion of the lacunae empty.

I determined the distribution of dead cells and live cells as shown by whether or not there was a sharply stained nucleus, and it was quite apparent at once that inside the haversian system, inside the cement line, usually practically all the cells are alive. By the very nature of things there must be areas outside all haversian systems (interstitial lamellae), and one immediately sees that the proportion of dead cells in those places is much higher than it is inside the haversian system.

The reason for this would seem fairly apparent. There are good



canalicular connections between all lacunae inside the haversian system, but across the cement line very few canaliculi pass; therefore, diffusion is difficult, these cells get cut off from their blood supply, and tend to die.

Now let me draw a contrast between haversian bone, such as is found in humans, where practically all bone is occupied with the haversian system, and what I call laminar bone (what Enlow and Brown (ref. 95) and others have called plexiform bone), such as is found in cattle, where there are a series of two-dimensional, flat networks of blood vessels with fairly large-bore channels connecting them (fig. 51). There is a very good blood supply in the plane of the networks, but the supply is not so good in the plane at right angles. This is very characteristic of large, hollow bones.

Another observation I made when looking at beef femurs is that if one looks at a single lamina, at the midpoint between the two blood vessel nets, there is what is called the bright line across which very few canaliculi pass, rather like across the cement lines in haversian systems. (See fig. 51.) This is not a reversal line. I found in cattle bone that it was rare to find only one haversian system in this primary bone. What one tends to find is several in a row, occupying a single lamina. This suggested to me that if for some reason a haversian system formed in a particular place in this laminar bone, the blood supply of the areas on either side of it in the same lamina could not be made up by blood flowing from the neighboring laminae. Therefore in the middle of this network, there would be a kind of cylinder across which no blood passed. This cylinder would tend to restrict the blood supply on each side. This seemed to be a reasonable explanation of the fact that these haversian systems occur in rows and usually extend up to, but not farther than, the bright line in the middle between two laminae.

So the morphology certainly makes sense if you have one haversian system forming, then you will be liable to have more haversian systems forming around it, simply because the first system interrupted the blood supply locally.

I looked at bone that was very healthy, that is, bone with very few lacunae without strong staining nuclei, but which had a few haversian systems forming and in which the outline of the cavity was irregular; there was no cement line.

I then looked to see if there were any dead cells in about 30 microns around the outside of this forming haversian system cavity; I also looked in comparable areas at random elsewhere in the section. The bone as a whole was very healthy; that is, dead cells were rare. I found that there was a significantly greater number of dead cells in the neighborhood of forming haversian systems than there was in the generality of the bone. The number of dead cells was not very high, but never-

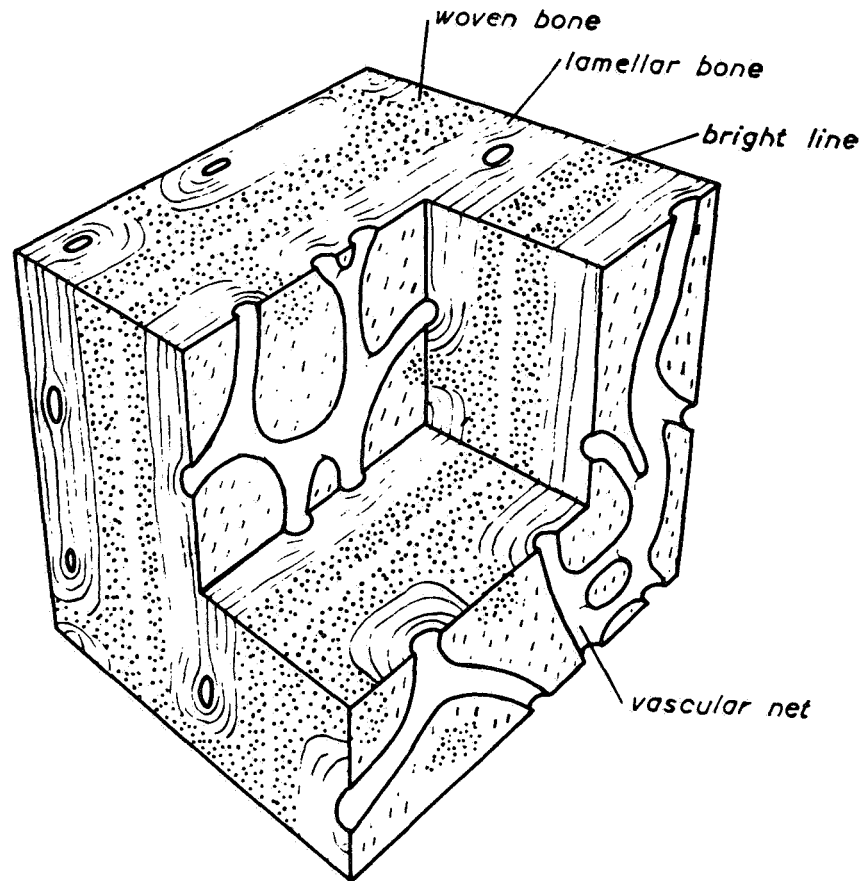


FIGURE 51. Block diagram of a small portion of laminar bone, showing parts of the vascular nets of two laminae.

theless it was significantly in excess around forming haversian systems than elsewhere.

Now, to what does one attribute this?

HEANEY: In what you have said, particularly with respect to the lamina of bone, it has been assumed that a poor vascular supply predisposes to haversian remodeling. Would you care to cite the evidence for this position?

CURREY: No, I would not; I would rely on commonsense. If you have no blood supply and no canalicular connection with neighboring cells, and if you are completely isolated except for what actually gets through the hard bony tissue, then it would seem to me reasonable to expect that these cells would be in metabolically a much less favored place than those cells that were in close connection with blood vessels

to which their canaliculi extended. This would seem to be common-sense. I have no evidence for this.

HEANEY: There is no question that, deprived of a proper blood supply, the cells would be compromised; but the question is, Does this predispose to osteoclastic resorption?

CURREY: Well, I do not know. I am just producing a series of observations which might indicate that where cells are in a bad way is where you tend to get haversian systems. To explain the observation where I looked at the healthy sections with a few dead cells and the dead cells tended to be around forming haversian systems, you might say it was the process of the forming of the haversian system which leads to cell death. This may be so.

I did another slightly finer analysis of this. I looked at rather small, forming haversian systems. Haversian systems have a fairly characteristic size in any part of a bone, and one could say, "Well, that has gone only about a third of the way." One can tell the size it is going to be before it starts. And I found the ones that were very small, that did not get very far, had dead cells surrounding them more frequently than those that had practically reached their full size; therefore, it is as if—and this is pure hypothesis—there is a blood channel in a primary osteon, not in a reconstructed osteon, and a dead cell nearby. This may cause the formation of a haversian system at that level. I may say that the sections studied were fairly thick and include in their length about the distance that Cohen and Harris (ref. 77) found tended to be remodeled at any time. Haversian systems do not form along a great length at one time. I thought it might be reasonable to suppose that where there are one or two dead cells, osteoclasia may in some way be stimulated in that region, and there will tend to be a haversian system formed at that point. Therefore, if you look at the small systems still forming, your chances of finding the dead cells outside the area are higher than if you got to the outermost level, where they would very probably have been resorbed.

It is true that the formation of an erosion cavity does not lead in any general sense to the destruction of cells; I very frequently saw nuclei half in and half out, just before it was incorporated into the central space.

Figure 52 illustrates the very characteristic kind of pattern which one can observe. Figure 52(a) is a haversian system with two blood channels, with an erosion cavity starting from one channel. Figure 52(b) is a later stage where old cells that were surrounding the central blood channel are now cut off from the new blood supply. The blood supply has gone sideways. You can follow it up for several generations quite frequently (fig. 52(c)). The cells were cut off when the new haversian system was formed.

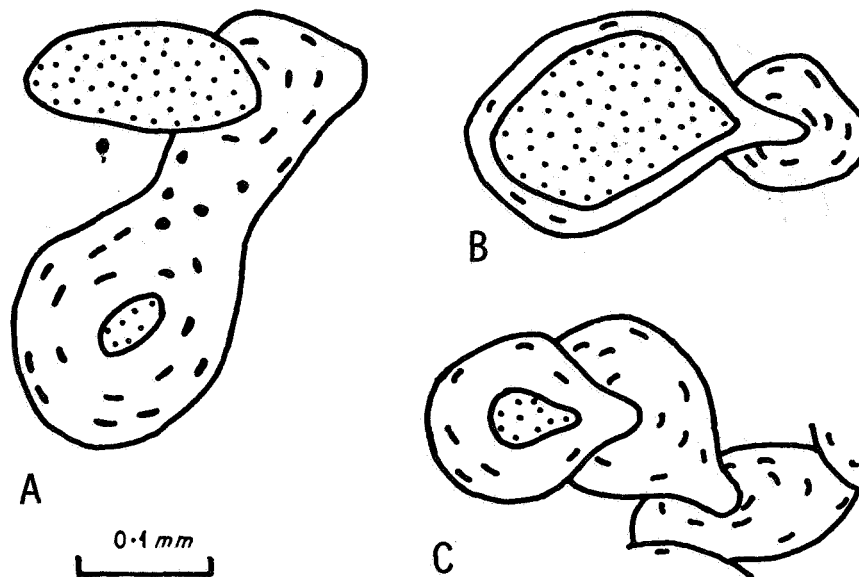


FIGURE 52. Three stages in the formation of haversian systems.

You can see the kind of logic in the morphology. It is apparent that these older haversian systems are going to be in a worse way than the new ones, and this certainly does seem to be the case if you look at the proportion of dead cells inside and outside the haversian system.

You might say, "Well, this is why haversian systems form in one place." It may be that one or two form, and this interrupts the blood supply and the general metabolic setup there; then there are more and more forming.

There are one or two things against this. In figure 53 are drawings of cat ulnae; the stippled area is primary bone, the white area is haversian bone, and the black areas represent forming haversian systems, right and left. The agreement of the places where the haversian systems are forming is rather remarkable. The areas of haversian systems are in almost exactly the same place.

Marotti in 1963 (ref. 96) has done this in more detail and gets practically the same picture. You almost get stereo pairs of the formation of haversian systems which, in this case, are not altering the external form of the bone at all.

Any suggestions as to why one should find haversian systems forming in exactly the same place? I do not think that it is because of the idea I have produced here, that is, interruption of the blood supply. It would be unlikely that one would obtain such a neat agreement.

HOLTZER: Could you do the obvious experiment of putting in something like skull bone as a graft? Do you think these haversian systems

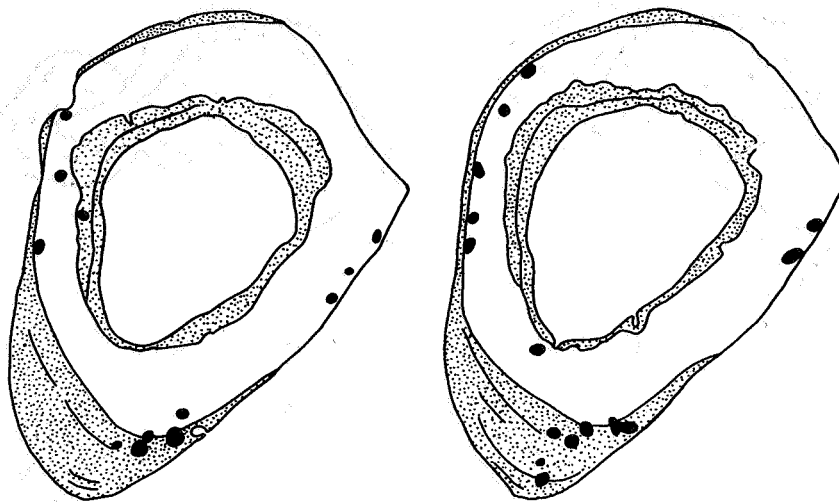


FIGURE 53. Camera lucida drawings of the right and left ulna of a cat. Stippled area is primary bone, white area is haversian bone, and black areas represent forming haversian systems.

would go right through a complex bone that does not have the usual haversian system? I want to know whether it is the whole area that is destined to be canalized by the haversian system, or is it something peculiar about the bone? By putting another kind of bone in there, could you get some information on what causes these deposits?

CURREY: You quite probably could. Of course, the trauma of actually putting in a graft and interrupting the blood supply to put in a graft would probably negate any virtue to it.

HOLTZER: Could you not come back to it 2 years later?

CURREY: Yes, possibly.

URIST: What is the external shape of the bone at the level of the section?

CURREY: Are you talking about the stress on the bone producing the gaps?

URIST: Were there convexities or concavities or neither of these in the three lines of section? Were you able to see anything on the external contour of the bone that would coincide with the localization of resorption cavities or overgrowths?

CURREY: I did not look specifically for that. I think any strain would be too small to be noticeable at this level. The stress would have to be pretty large to produce local humps like this.

PECK: How about insertions?

CURREY: In the linea aspera, for instance, there is much more reconstruction under the insertion. This is even more marked in cattle,

where the homolog of the linea aspera is usually the only place where you get any large amount of reconstruction in the femur.

I took an 8-year-old cow, which was called by the farm "a good milker," because I felt that this cow might be under stress as regards calcium. If there is any stress, those giving a lot of milk might be expected to show quite a lot of haversian remodeling, and there was not a trace of it except under the muscle insertions.

RAISZ: Do they resorb in this laminar area?

CURREY: No; the only way to get resorption in a laminar area is to form a haversian system in the ordinary way.

WHEDON: Have you looked at bones from animals of different ages? Is there a difference with age in the formation of the haversian system?

CURREY: I have looked at young cattle and old cattle; there is no alteration.

WHEDON: The reason I asked this question is that Smith and Walker (ref. 97) have shown that in long bones as they age the cortex becomes thinner, but the actual diameter of the bone increases because the bone is sort of expanding as it ages. One might expect that there would be some indication by one method or another of remodeling along the outer surface rather than along the inner surface.

CURREY: Yes, but I am talking only about reconstruction that takes place entirely within the cortex of the bone. I think what goes on in the surfaces is another matter.

You do get changes in the mode of reconstruction with age. Older people form smaller haversian systems (ref. 98). They have more and smaller haversian systems than younger people.

ROWLAND: Before you go on, may I interrupt for a moment? You referred to the work by Marotti (ref. 96)—he also showed that haversian remodeling occurred in similar locations when pairs of long bones were examined at identical levels. But there is a fourth dimension involved. You are looking at a two-dimensional representation of a three-dimensional section; the fourth dimension is time. Should we not expect that the number of such sites will change as the animal ages?

CURREY: The number of what?

ROWLAND: The number of resorbing areas or rebuilding areas; that the number, the gross number, was a function of the animal's age.

CURREY: Yes.

ROWLAND: Does this not tend to eliminate the possibility that the raw number is a function of stress?

CURREY: If the quality of the bone changes through, for example, increased mineralization so that it becomes stiffer, the bone may have a greater or lesser strain to put up with. I do not know. I do not mean to eliminate this. I am not putting forth the idea of strain particularly. I am not offering any ideas as the only answer. I am utterly confused.

Now I would like to continue and to ask whether haversian systems are a good thing from a mechanical point of view. This is, more or less, the first experiment I did when I started working on bone (ref. 99). I obtained a whole series of samples of beef femurs, tested them in a tensile testing machine, and correlated the strength with the known percentage of haversian systems; the answer was quite straightforward. The more haversian bone, the weaker is the bone in tension.

This work is not being repeated exactly, but a complementary study has been done recently by Heřt et al. (ref. 100); they did not get a nice clear answer such as I did because they employed a different method of plotting. They talked about bone that is almost entirely haversian, on the one hand, and all the rest of the bone, on the other hand, so they have lumped a great part of my distribution. But they certainly did not find that the haversian bone is stronger than primary bone.

Therefore, the idea that haversian remodeling makes a bone stronger in tensile strength is not a good one.

PRITCHARD: It is not a good one? Would you repeat the suggestion that there are a lot of these haversian systems which are not fully calcified and that this is why haversian bone is weaker than other bone?

CURREY: I would not want to repeat this, because at this point I am not worried about why haversian bone is weaker than other bone. But when I was taking the specific tensile strength, I made allowance for those haversian systems that were still in the presence of forming by reducing the cross-sectional area of the whole bone by a proportional amount up to the inside of the collagenous bone; but I do not think it will be very significant. If it were significant, this may indicate why haversian bone is weak.

PRITCHARD: It may take 3 months to complete the calcification of a haversian system after all its organic matter has been laid down.

CURREY: Yes. And if one wanted to argue, as people have, that the fact of making haversian bone reduces the strength of the bone part of the time, as it were, this is an argument against this.

Now, against the idea that the location of haversian systems is not related to mechanical needs of the body is the localization not within a bone but between bones.

I took a cat and observed the percentages of haversian bone in various parts of its body (table VI). I compared the long bones with the vertebrae and pelvis, and found that the average amount of remodeling, or haversian bone, in the long bones was about 50 percent and that the average amount in the vertebrae was 87 percent. In other words, much more remodeling had gone on in the compact bone—I am not talking about the spongy areas—of the axial skeleton than in the long bones. This makes good sense mechanically, because if you had a bone of infinite size it would be infinitely strong, but also infinitely

TABLE VI  
PERCENTAGES OF HAVERSIAN BONE IN THE CAT SKELETON <sup>a</sup>

Bone	Upper	Middle	Lower
Humerus.....	40	55	35
Radius.....	50	55	70
Ulna.....	80	65	60
3d metacarpal.....		50	
3d digit, 1st phalanx.....		70	
Femur.....	40	25	40
Tibia.....	65	60	60
Fibula.....	70	0	45
3d metatarsal.....		55	
3d digit, 1st phalanx.....		50	
1st thoracic vertebra:			
Neural spine.....		85	
Centrum.....		85	
Rib.....		85	
7th thoracic vertebra:			
Neural spine.....		90	
Centrum.....		80	
Rib.....		95	
7th lumbar vertebra: Centrum.....		90	
1st sacral vertebra.....		85	
Pelvis:			
Anterior.....		90	
Posterior.....		80	
Mandible.....			65 (total)
Petrosal.....			0 (total)
Parietal.....			0 (total)
Squamosal.....			55 (total)

<sup>a</sup> The percentage of the total area occupied by haversian bone in sections from different sites from the skeleton of a mature cat.

heavy. Therefore, you must have a compromise between the weight of the bone and its strength, and you have to allow some kind of safety factor.

When an animal is running along you can make any part heavier or lighter, as you wish. If you make the spine heavier, then you just increase the speed at which it can run by some simple function of its weight. If, however, you make a long bone heavier, you decrease the efficiency of the running by some power of the increase in weight because the legs are moving back and forth; you have to consider the moment of inertia of the distal parts of the long bones, and these are some function of the weight and the square of distance.



I am putting this badly, but what I am getting at is that you can afford the safety factor in the spinal column; for example, when horses fall down and break their legs, they usually do not break their spines. I think this is significant, because the horse is highly adapted to very fast running. The horse clearly has not a great safety factor in its legs.

Therefore, you can say, "If you are going to remodel bone and make a horse run faster, then for heaven's sake do it in the spine, where you have a high safety factor"—that is indeed what one finds.

One could also say, "All right, but remodeling is to provide for the mineral needs of the body. When you use calcium internally, you have to tear some out of the bone and put it back later." This may be so. It does leave a lot of problems. For instance, why remodel within the shaft of the long bone? Why not do it simply at the endosteal border? Because, clearly, if you have a shaft of a long bone which is subjected to bending, the part that needs to be strong is the subperiosteal area around the outside, and the bone becomes of diminishing mechanical importance until you can get into the endosteum. Essentially, the obvious place to get rid of bone is along the inner border. I know, in fact, that there is less remodeling on the outside than the inside. But why is not all remodeling on the inside?

If the purpose of remodeling were the mineral metabolism and if I were Dame Nature, I would put a lump of bone somewhere in the pelvic region, or in some cavity which had no mechanical function whatsoever, and just let that be taken away from or added to as was required.

This does not happen. We use good functional bone, and I think the primary purpose of bone is to enable you to walk around; then you start weakening it by remodeling it.

Another difficulty is that if the purpose of remodeling is to make up the calcium level of the blood, it is strange that there is less remodeling in very small mammals like mice. In these small mammals, the ratio of weight of the skeleton to the weight of the animal as a whole, and presumably of the body fluid, is lower. The bones of a mouse are relatively small, and yet the bones of a mouse do not show haversian systems even though many of them are large enough to do so. Yet these bones have relatively a much larger body of body fluids to support, so that is another difficulty.

Then there is also the strange distribution of haversian systems among the mammals; these systems are found much more in primates and in carnivores than in herbivores. I cannot produce an explanation for this. I cannot see why western Europeans and North Americans, who presumably have a pretty easy time of it from the point of view of their calcium and phosphorus, have a vast amount of remodeling; this remodeling is not simply a function of getting older because bone

starts to be remodeled in humans almost as soon as it is formed. I do not know to what extent we start going into lowered calcium intake; but not very often, I would think.

RAISZ: May I go back for a minute? I think I got lost in something. The idea you gave me before, that haversian remodeling occurred in a bone at a site which was poorly vascularized and had perhaps, therefore, dead cells, would lead me to think that the next thing to test is the tensile strength of the dead bone that you are replacing with the haversian system, rather than the healthy bone around it. That may be impossible to do, but at least you can give us a guess about it.

CURREY: Yes. I think it will probably be lower. I was simply comparing haversian and nonhaversian bone, and did not analyze this. I think this would be possible to do. That is a good point; haversian bone might be making the best of a bad job. Why the bad job is there in the first place is, of course, another matter; I am suggesting that it might be a bad job simply because this is a rather chicken-and-egg thing.

What I have been trying to do is to show that if you look at this from the point of view of the way natural selection works, there are many ways of looking at remodeling. It would seem to me that where the remodeling actually takes place is probably, to some extent at least, determined by where remodeling has already taken place; once remodeling goes on at one place, then, because of the interruption in metabolic processes going on, you are liable to get it going on in that place as well.

MACDONALD: Are there not some stresses other than tensile for which the haversian system is advantageous, just twisting stresses?

CURREY: When you get a torsion fracture, the bone material rather than the femur as a whole has broken in tension. You get a shearing stress that resolves itself into a tension stress at  $45^\circ$  to the direction of the shear. Most bones, of course, are loaded in compression, but the reason that bones actually fail is that they get bent; the part that goes first is the part that is on the convex surface, the part that is under tension.

I think tension is the meaningful stress to consider even though what happens to the bone as a whole may be much more complicated. It may be bending; it may be torsion.

MACDONALD: Was it not the idea that this makes a stronger tube; i.e., the explanation of the importance of the change in direction of the collagen fibers in adjacent lamellae?

CURREY: Yes. That makes it stronger in various directions. You get this in laminar bone. One bit of laminar bone is really like a haversian system split open and spread out. You get this different orientation of collagen fibers.

HEANEY: You indicated that you thought bone had evolved in order to enable us to walk around. Dr. Urist has some thoughts on that.

URIST: The earliest vertebrates had bone only in the exoskeleton. It was dermal bone and used for armor in bottom-dwelling fishes, the ostracoderms which were subject to hydrostatic pressure on all sides. The metabolic activity of exoskeletal bone tissue is probably comparable with that of teeth. After a survey of the microscopic anatomy and the chemical composition of the blood of some representative living lower vertebrates, it occurred to me that the skeleton and the body fluids react as if they were part of a single system. In the course of time and evolution, bone functions as a servo unit, first for an open- and then for a closed-cycle system. Figure 54 (ref. 101) summarizes in diagrammatic form the observations we have made on bone-body continuum.

The earliest forms depended upon the sea for storage of minerals. The reduction in calcium in the blood plasma from 44 to 22 milligrams per 100 ml came at the parting of the ways between marine invertebrates and primitive marine vertebrates. The use for internal control mechanisms and storage of important minerals, such as calcium and phosphorus, decreased total-ion and calcium-ion concentrations of the body fluids. The skeleton became more cellular and capable of active turnover of the body stores of calcium and phosphorus. The first amphibians ventured on land some 300 million years ago when the skeleton contained true bone cells; the body regulatory mechanisms included vitamin D, the parathyroid hormone, as well as the more primitive membrane control, and tissue protein binding of essential ions.

Table VII lists the organs used for homeostasis of calcium ion from the lowest to the highest vertebrates. In the cyclostomes there is no apatite mineral in the skeleton; the supply in the external environment, however, is unlimited. Ionic equilibria are sustained by skin, gill membranes, gut, and kidney by means of an open-cycle system. The serum concentrations of calcium are maintained by mechanisms that act as an ion pump in fresh water and an ion dam in sea water to control unidirectional flow, or movement of ions too rapidly toward the concentration gradient. Hormonal control of calcium metabolism does not appear until the evolution of bone or the storage unit for a closed-cycle or servo system for regulating the turnover of phosphate and calcium ions. Vitamin D appears first in the teleost fishes, and the vitamin D-parathyroid hormone synergism appears first in the amphibians, when the vertebrates came out of the water on to the land.

The elasmobranchs store large quantities of vitamin A in the liver. The teleost fishes that live in a marine habitat do not utilize vitamin D

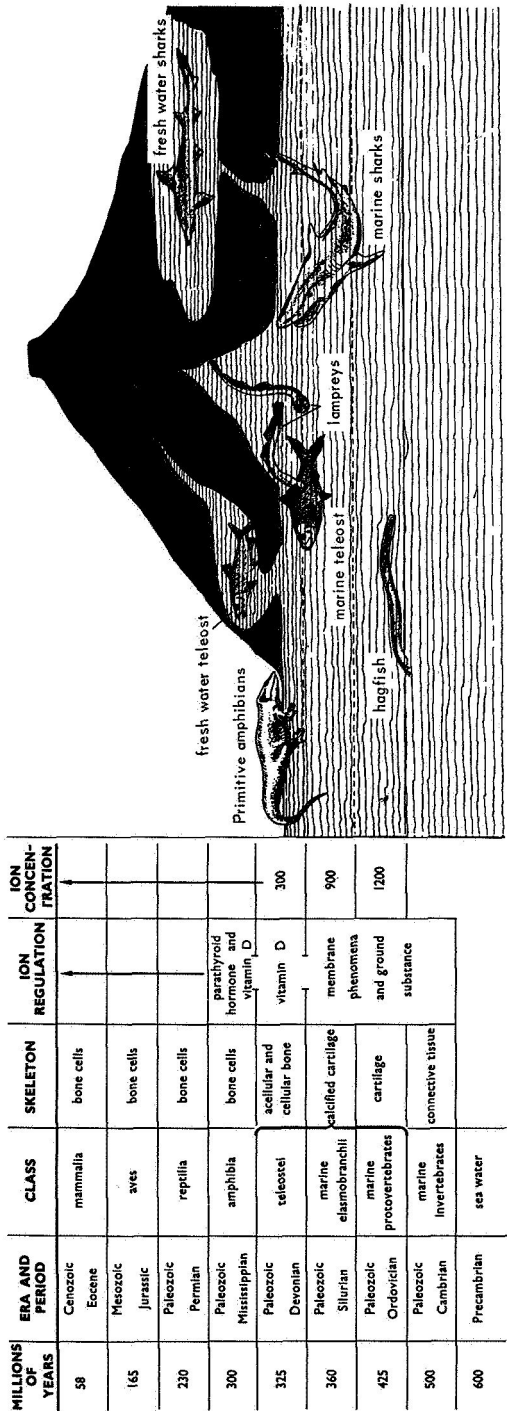


FIGURE 54. Diagram of relationship between the mechanisms of ionic regulation, ion concentrations of the blood, compositions of the skeleton, and differences in the external environment of various classes of vertebrates. [From ref. 101; reprinted by permission of the publisher.]

TABLE VII  
HOMEOSTATIC MECHANISMS FOR CALCIUM AND RELATED IONS IN THE FLUIDS OF THE BODY

Vertebrate	Integument	Skeletal system	Respiratory system	Endocrine system <sup>a</sup>	Kidney	Liver (vitamin)	Intestinal tract
Mammals and birds.....	Skin.....	Bone and cartilage.....	Lungs.....	P, T, G, A, PTG.....	Metanephros.....	A, D.....	Gut.....
Reptiles.....	Scales.....	Bone and cartilage.....	Lungs.....	P, T, G, A, PTG.....	Metanephros.....	A, D.....	Gut.....
Amphibians.....	Mucosal skin.....	Bone and cartilage.....	Lungs.....	P, T, G, A, PTG.....	Mesonephros.....	A, D.....	Gut.....
Teleosts, fresh water.....	Scales.....	Bone and cartilage.....	Gill membranes.....	P, T, G, IRB, UBB.....	Mesonephros.....	A, D.....	Gut.....
Teleosts, marine.....	Scales.....	Bone and cartilage.....	Gill membranes.....	P, T, G, IRB.....	Mesonephros.....	A.....	Gut.....
Elasmobranchs.....	Scales.....	Calcified cartilage.....	Gill membranes.....	P, T, G, IRB.....	Mesonephros.....	A.....	Gut.....
Lamprey.....	Mucosal skin.....	Cartilage: no mineralized tissues.....	Gill membranes.....	AH, T, G, IRB.....	Pronephros; many nephrons drain from each segment.....	A.....	Gut.....
Hagfish.....	Mucosal skin.....	Cartilage: no mineralized tissues.....	Gill membranes.....	AH, T, G, IRB.....	Pronephros; single pair of nephrons in each segment.....	A.....	Gut.....

<sup>a</sup> P—pituitary; T—thyroid; G—gonads; A—adrenals; PTG—parathyroid glands; IRB—interrenal bodies; UBB—ultrabranthia; bodies; AH—adenohypophysis.

but store it in the liver. However, when teleosts come into fresh water, their liver stores relatively little vitamin D because it is used for turnover of calcium.

The sturgeon (fig. 55) is an interesting vertebrate in that it is phylogenetically deficient in vitamin D; it has physiologic hypocalcemia. Nearly all of the bone is on the outside of the body in the form of scales, and the calcium in it is probably about as unavailable as the mineral in teeth (fig. 56). Figure 57 is a cross section of this sturgeon showing that the only endoskeletal bone tissue is the bit of tissue at the base of the skull. The blood calcium may be only 7 to 8 mg/100 ml, but the total-ion concentration is the same as any other vertebrate (table VIII). Vertebrate paleontologists say that the sturgeons throughout the course of evolution have lost bone; they contend that fossil sturgeons had lots of bone in the skeleton. The parathyroids presumably came from the ultimobranchial bodies. Apparently the ultimobranchial bodies, the gill arches, and the gills themselves, as Dr. Copp suggested, have something to do with the evolution of both thyroid and parathyroid.

WHEDON: What is the alkaline phosphatase?

URIST: The level of alkaline phosphatase is the same as in any other vertebrate.

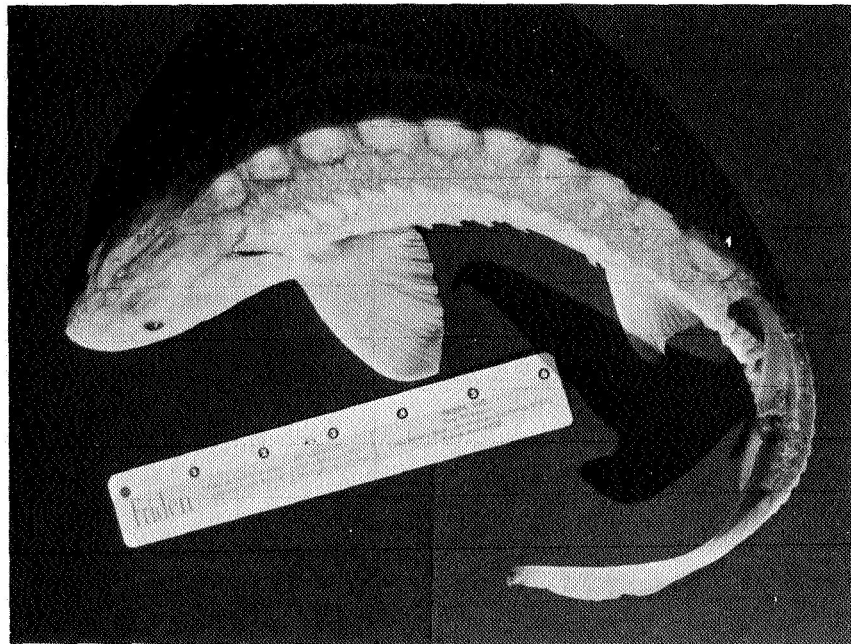


FIGURE 55. Photograph of the young sturgeon, *Acipenser medirostris* Ayes.

I think that in the story of the evolution of bone and calcium metabolism, the parathyroid hormone plays a role in calcium homeostasis with the development of finer regulation. These hormones appear in vertebrates that exhibit very high-speed swimming, rapid movements, high-energy phosphate metabolism, and terrestrial life. The regulation of the level of calcium, to about 7 or 8 mg/100 ml, in the fluids of the body can go on without vitamin D and without parathyroids. Where thyrocalcitonin appears in the scale of evolution remains to be determined by bioassay of the thyroid of lower forms.

BUDY: How does the haversian system fit into the story?

URIST: Haversian systems may be the mechanism of replacement of aged bone cells by new cells, with or without any relationship to the mechanisms of calcium homeostasis. Once the formation of a resorption cavity gets going, it has no respect for bone; that is, whether the cells are dead or alive. Perhaps the stimulus for resorption comes from an area of one or two dead osteocytes.

The permutations and combinations of factors associated with bone formation and resorption are enormous. Instead of trying to find one



FIGURE 56. Roentgenograph of a sturgeon showing partial armor of rows of plain bony plates. There is very little bone in the endoskeleton, and only in orbital and auditory regions, and the posterior end of the base of the skull. The relationship between the endoskeleton and the exoskeleton is shown in cross section in figure 57.

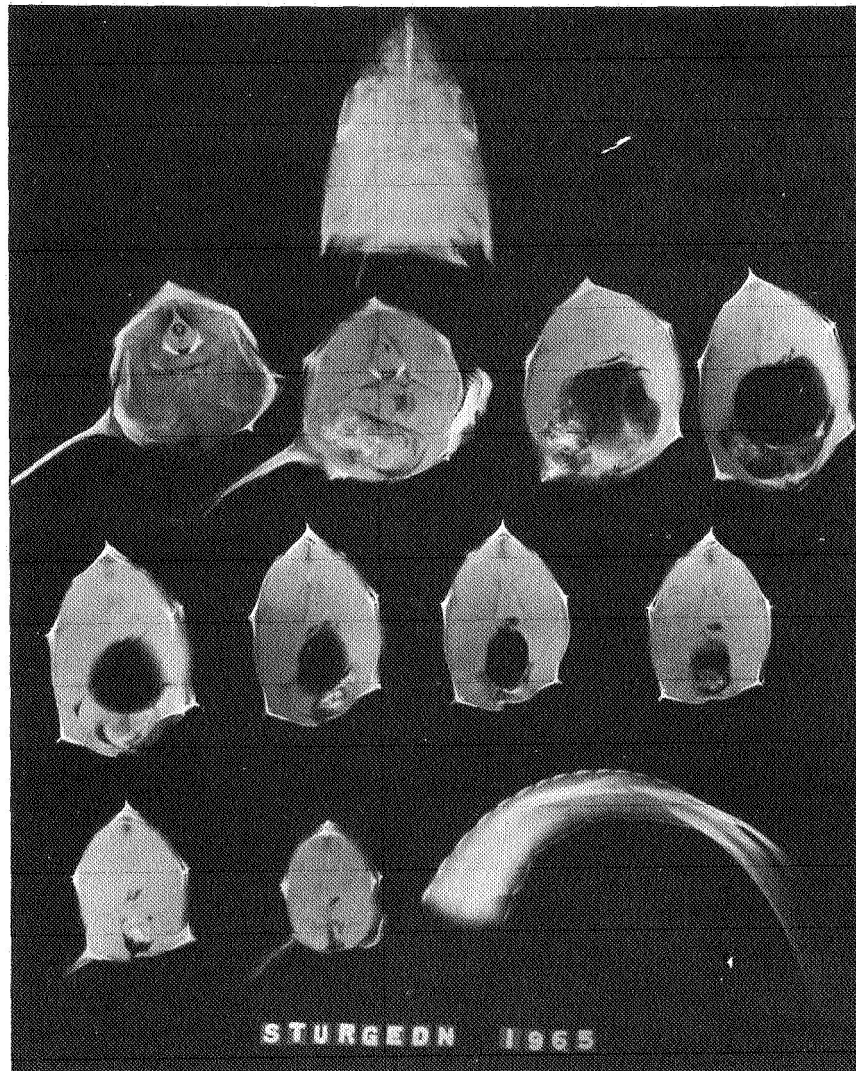


FIGURE 57. Roentgenographs of cross sections of the entire length of the sturgeon shown in figure 56. Note the location of deposits of calcified tissue in the base of the chondrocranium, but none elsewhere in the endoskeleton. The bony plates in the exoskeleton encase the entire body in armor.

explanation, one should look for a combination of factors, both physical and chemical.

HEANEY: Dr. Currey has indicated that he is a zoologist and tends to look at things from the point of view of a naturalist. Would anyone care to hazard an estimate of the selective advantage conferred upon



TABLE VIII

CHEMICAL COMPOSITION OF SERUM OF STURGEON, *Acipenser transmontanus*, IN MARINE AND FRESH WATER HABITAT

Component	Males in marine habitat (San Pablo Bay)	Males and nongravid females in fresh water (Suisun Bay)	Gravid females in fresh water
Sodium.....mmoles/l.	130.0± 15.0	129.0± 11.9	123.0± 10.7
Potassium.....do.....	2.5± 0.4	2.7± 0.5	2.0± 0.3
Calcium.....do.....	1.7± 0.4	1.8± 0.4	4.6± 0.4
Magnesium.....do.....	2.1± 0.2	2.0± 0.4	1.1± 0.1
Chloride.....do.....	115.1± 7.1	111.0± 6.7	116.0± 12.4
Bicarbonate.....do.....	5.2± 0.2	6.0± 0.8	5.0± 0.5
Sulfate.....do.....	0.4± 0.1	0.5± 0.2	0.2± 0.1
Phosphate, inorganic.....do.....	2.9± 0.4	3.3± 0.4	4.1± 0.6
Total.....	259.9± 34.0	256.3± 30.0	256.0± 34.0
Urea nitrogen.....mg/100 ml.	2.8± 0.1	2.5± 0.2	3.0± 0.2
Citric acid.....do.....	0.3± 0.1	0.4± 0.1	0.8± 0.1
Albumin.....g/100 ml.	1.6± 0.1	1.5± 0.1	3.0± 0.4
Globulin.....do.....	1.0± 0.1	1.0± 0.2	1.0± 0.2
Total protein, g/100 ml.....	2.6± 0.1	2.5± 0.2	4.0± 0.4
Alkaline phosphatase, units.....	2.9± 0.3	3.0± 0.4	3.1± 0.4

vertebrates by haversian systems? Obviously, this is a very primitive affair. It looks as if nature is trying to tell us something here, if we could read her handwriting.

BUDY: I remember some microradiographs of dinosaur bone that Jenifer Jowsey had (unpublished data). As I recall, there were many osteons and resorption cavities. Dr. Currey, have you compared dinosaur bone with bone from other species?

CURREY: Dinosaur bone is just like cattle bone. I obtained some dinosaur bone because I was interested in whether, in fact, haversian systems turn the primitive laminar blood supply into a more efficient system, and I plotted the distance of random points from the nearest blood vessel in laminar bone and haversian bone in the same femur. I did exactly the same exercise on fossil dinosaur bone and found the same answer (ref. 102).

BUDY: What about the dugong and the manatee? They also have many osteons and resorption cavities (J. Jowsey, unpublished data).

FREMONT-SMITH: Do they have haversian systems?

URIST: The manatee has haversian systems but, in a relative way, it has a paucity of resorption cavities. Practically all of the bone that is laid down just stays there. The marrow cavities are almost nil. It has what is comparable to osteopetrosis, but not exactly the same thing. It should be called pachyostosis.

HEANEY: Unresorbed calcified cartilage?

NICHOLS: It is pseudohypoparathyroid, really.

URIST: The bones of the adult do not contain large pockets of unresorbed calcified cartilage as in osteopetrosis, but I have not seen the bone of the newborn manatee or dugong.

NICHOLS: I would like to propose an alternative to Drs. McLean and Currey's idea that haversian systems are valuable from the point of view of strength. I think one can postulate another use for them, namely, that haversian systems provide us with a constant supply of new mineral. In support of this just the thinnest thread of data may be cited. We know that as the mineral gets older, the crystals pack a little better, and the strains within them get a little less. We also know from our crystallographer friends that under these circumstances the time and/or the stimulus required to dissolve a given amount of mineral is greater if the mineral is older than if it is younger.

The mouse and the rat, which do not have haversian systems but have pretty good calcium homeostasis, probably use their gut as a potential pool for readily available calcium, as has already been pointed out. Man, however, does not seem to use his gut in this way, but he has very good—indeed probably far better—control. I suspect that man uses his haversian systems as the very rapidly available pool which is needed for such close control.

Dr. McLean says that man has enough other machinery to supply him with the necessary calcium to maintain his serum calcium concentration. This may be true, but I suggest that the newly calcified layer in the newly formed haversian system (in which mineralization is somewhere between 70 and 90 percent of the maximum possible) is the area where there are fresh crystals; where a very small amount of acid poured out will dissolve a lot of mineral in a very short time; and that this area, indeed, is the site where the homeostatic control is really exerted.

HEANEY: That seems all very right, Dr. Nichols, and it makes it very nice that we have haversian systems, but it gives a good bit of credit to the intelligence of the osteoclast. How does the osteoclast know that the organism is getting short on fresh bone and should tunnel out some of this old compact bone so that it can make some more fresh bone?

NICHOLS: May I go on with my theory? I think Dr. Currey has that answer. The osteoclast smells a dead-bone cell.

URIST: If that is true, an osteoclast may function by the same intracellular mechanism as a macrophage or a foreign body giant cell.

HEANEY: I was hoping someone else would say that so I would not have to.

COPP: May I suggest another rather nebulous hypothesis for the distribution of haversian systems? The animals in which you find well-developed haversian systems are carnivores and primates, which normally feed on a diet high in phosphate and low in calcium. This would tend to depress plasma calcium, to stimulate the parathyroids, which in turn would stimulate osteolysis. On the other hand, herbivores normally eat a diet low in phosphate, which would tend to produce hypercalcemia and increased thyrocalcitonin production, with reduced osteolysis.

CURREY: Some such explanation would seem very reasonable. One can check this by looking at primates and carnivores with odd diets. I suppose termites would be high phosphate, would they not?

COPP: High in phosphate no doubt, but rather low in bone.

CURREY: What is a leafy diet?

COPP: A leafy diet is high in calcium and low in phosphate (Ca/P ratio is 2/1 to 5/1) while a meat diet is just the opposite (Ca/P ratio is 1/10 to 1/20).

CURREY: Well now, the colobus monkeys eat leaves almost exclusively, while other monkeys eat nuts and fruits; it would be extremely interesting to compare these two types of monkeys.

PRITCHARD: The herbivores tend to be eating all the time; their stomachs are always full. The carnivores and the higher primates tend to eat three meals or less a day and have long periods of fasting. Homeostasis obviously is more of a problem for these animals than for those that have their belly full all the time.

COPP: Our sheep, for example, are ruminants and absorb nutrients from the rumen almost continuously. It is as if they were fed a continuous intravenous drip, and one must fast them many days to get any sign of starvation.

HEANEY: Dr. Copp, these haversian systems in bone remodeling continue to be present in the absence of the parathyroids, do they not?

COPP: Yes; but osteolysis and remodeling can take place in the absence of the parathyroids.

HEANEY: However, one does not need to interpose the parathyroid, even though it may have an effect.

TALMAGE: I would say one would. The system that the parathyroid might stimulate, which inaugurates the formation of resorption cavities in compact bone, should also work in the absence of the

parathyroid, although at a reduced rate. As an example of this, osteoclast numbers in the metaphysis can be increased by lavaging a rat with a calcium-free rinse. If the animal is parathyroidectomized, this effect is essentially, but not completely, negated. Even in the absence of the parathyroid, stimulus for osteoclast formation remains but is considerably reduced in activity. This should apply also to compact bone where the formation of osteoclasts is the stimulus for the start of a resorption cavity.

NICHOLS: But there is no evidence so far that there is more haversian remodeling in chronic hyperparathyroidism, is there?

TALMAGE: I have no evidence myself, but a year ago I listened to Harold Frost arguing with Lent Johnson for an entire week. It was my impression that both men believed that there was a direct relationship between parathyroid activity and resorption cavities found in compact bone. Dr. McLean does not agree with this, and as I am not an authority I will not push the argument.

COPP: I agree with your point, Dr. Talmage, but it could be that carnivores do have this mechanism; this is the important point. It may be an adaptation to the type of diet.

HEANEY: Dr. Currey has raised a point about osteoclasts being stimulated by, aggravated by, or polarized toward dead or dying cells, and the question of a macrophage function has come up. There are obvious analogies here.

URIST: And the multinucleated giant cell as well.

HEANEY: I wonder if anyone would care to elaborate on this, because it seems to be a crucial point.

URIST: The multinucleated foreign body giant cell resembles the osteoclast both histologically and histochemically so much so that Irving and Handelman (ref. 103) in 1963 were not able to find significant differences between the two. Acid phosphatase, cytochrome oxidase, succinic dehydrogenase, and four other enzyme activities were exactly the same.

HEANEY: Would the cell kinetics people here care to make any comment about the origin of osteoclasts relative to the origin of macrophages?

BÉLANGER: Last summer we ran a short series of experiments, and we obtained a large number of foreign body giant cells in the granuloma pouch, as obtained by Selye's method (ref. 104); when we labeled these animals with radioactive thymidine and counted the number of nuclei that became incorporated within a given time inside these cells as compared with those which formed the osteoclasts, the number of nuclei was the same. The mechanism of formation of these cells appeared to be exactly the same, except that they were away from bone. That was the only difference.

URIST: Dr. Currey's suggestion that dead osteocytes incite bone resorption is supported by our observation on aged women with severe osteoporosis (ref. 105). On the basis of measurements of the thickness of the cortex of the femur, we estimated that aged osteoporotic women had lost 50 percent of their bone mass. Of the remaining 50 percent, half of that mass was dead bone. Dead bone was determined on the basis of cell counts. We wondered whether patients with senile osteoporosis resorb so much bone in an attempt to renew dead bone. The deficit in bone mass is not made up because the capacity to replace lost bone is apparently low in these aged ladies.

BÉLANGER: Also, if we look at sites of bone where foreign elements have become incorporated, these are the sites where a large number of osteoclasts are found, because the bone is abnormal. Wherever there is abnormal bone, which is a result of the cells dying out, or bone malformation as in rickets, or bone that has incorporated unnatural elements, then there are all kinds of osteoclasts.

FREMONT-SMITH: Would you get haversian canals at that time in those areas?

BÉLANGER: I am talking about surfaces in animals that do not have haversian systems.

URIST: Dr. Bélanger, do you regard Neutral Red as a specific stain for osteoclasts?

BÉLANGER: I do not know what part of the osteoclast you want to stain, but certainly it is easy to distinguish an osteoclast from a dead piece of bone if we use, for example, the Wright stain combination that I mentioned this morning. Bone will pick up the acid dye, eosin, whereas the osteoclast will always pick up the blue or azure component; it is very easy to see the osteoblast as well. Some people (including myself) have expressed the opinion that osteoclasts were just chunks of detached dead bone, but it is very easily shown that they are not, just by that simple method of staining.

BUDY: Dr. Bélanger mentioned the relationship of abnormal bone and osteoclasts. I think giant cells can also be included.

When one administers parathyroid extract to an incisor-absent mutant rat, as shown in figure 58, a marked change in the cell population occurs (fig. 59). There is a rapid mobilization of cells; osteoclasts are considerably larger than those observed in the untreated incisor-absent rat. They have many nuclei, and often one sees large vacuoles in the cytoplasm. The change is quite rapid, as though the stimulus for bone resorption caused relatively quiescent cells to become active resorbers.

In addition to the osteoclasts there is another type of cell, which I will call a giant cell for want of a better name. These cells have from 10 to 15 nuclei, are basophilic, but are not always adjacent to bone



FIGURE 58. Photomicrograph of a section of the tibia from an *ia* (incisor-absent) rat, 5 weeks old. The elongated spongiosa is one of the characteristics of this mutant strain of rats. Osteoclasts are present, but are somewhat inactive since resorption does go on, but at a reduced rate. Formalin fixation; HEA stain. 25  $\times$ .

spicules. They are present along the endosteum and can also be seen in the metaphysis where widespread resorption of trabeculae has taken place.

These are not macrophages; their location and appearance exclude the possibility of their being megakaryocytes, which are numerous in these rats. The giant cells do not have a polymorphous nucleus characteristic of megakaryocytes, but contain many distinct nuclei. With HEA stain these cells are basophilic and are not granular; but they do have many vacuoles in the cytoplasm.

Ordinarily, there are many variations in osteoclasts in these mutant rats; however, one does not see so many nuclei until the animal is

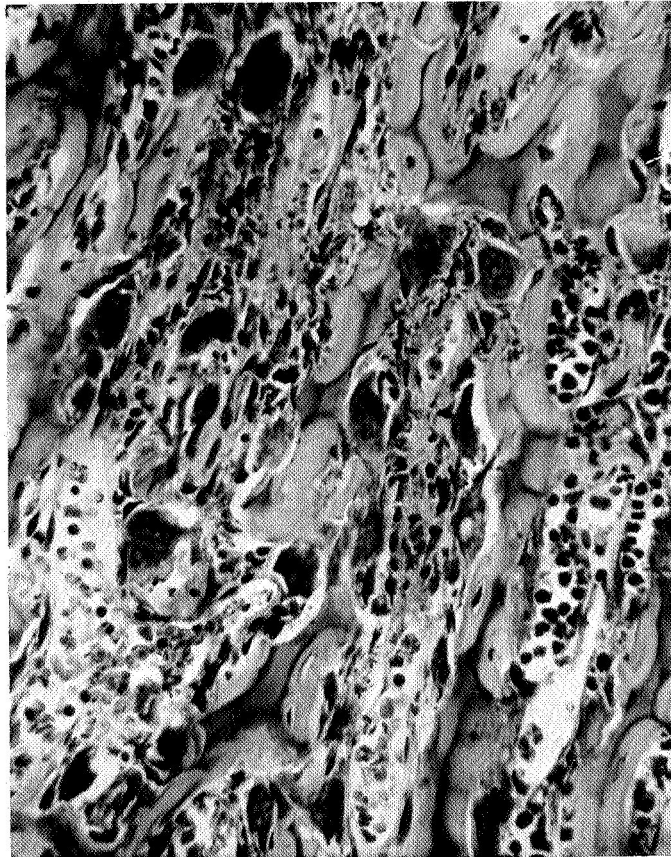


FIGURE 59. Photomicrograph of a section of the tibia from an *ia* rat following administration of 400 units of parathyroid extract (100 units daily for 2 days, then 50 units for 4 days). The littermate of this rat is shown in figure 58. Note the osteoclasts surrounding spicules of bone. After the spicules have been resorbed, these cells remain in the marrow and along the endosteum. Formalin fixation; HEA stain. 250  $\times$ .

stressed with PTE, for example. The picture is very different from that seen in a normal, nonmutant intact rat after PTE administration. (See figs. 108 to 112.) If these giant cells are osteoclasts, then they have assumed a new form to enable them to control their environment efficiently under stress. Once the initial stress is over, they continue in the same form and remain in fibrotic areas or in the marrow where bone spicules might have been present.

BELANGER: What is the treatment here?

BUDY: Lilly's parathyroid extract, 400 units over 6 days.

NICHOLS: Dr. Budy, did they get hypercalcemic?

BUDY: We did not collect blood on this group. I do not know.

ARNAUD: If you give them enough purified parathyroid hormone, both a calcemic and a phosphaturic effect can be observed.

URIST: There are at least two schools of thought on the origin of osteoclasts; one is the idea of Jee and Nolan (ref. 106) that the osteoclast can come from a fusion of macrophages, and the other is the idea of Fischman and Hay (ref. 107) in which the origin of nuclei of an osteoclast of a salamander is traced as far back as the monocytes of blood. Apparently, the question of cell origin depends on how far back into the pool of the cells of primitive mesenchyme one wants to go. One can suppose that every specialized connective tissue cell in the body can come from either a monocyte or a mesenchymal cell.

BÉLANGER: They come from primitive cells, osteoprogenitors, as Dr. Young calls them.

URIST: Yes; the progeny of a monocyte may enter the cycle of bone cells and become an "osteoclastoprogenitor" cell.

HEANEY: We have said nothing about blood supply except as it relates to cell nutrition. Dr. Whedon, do you have any thoughts on this?

WHEDON: No. I have a question. I have been confused through the years as to the effects of venous ligation, sympathectomy, and interference with arterial supply on bone. In some cases there seems to be increased formation and in other cases, increased bone resorption; I wondered if anyone here had some modern and reliable information on the effect of circulation on bone reconstruction.

HOLTZER: I would like to repropose the experiment that I mentioned earlier. Why could you not take something like a mouse or a chick and graft a lot of bone to its muscle, then let 1 or 2 years go by? I am sure you would have a lot of ectopic pieces of bone sitting around. Then look at the haversian system——

HEANEY: Is this the organ you are speaking about, this nonstructural calcium mass embedded in tissue which would be available for homeostatic purposes?

URIST: The crayfish has that—the gastrolith. The crayfish makes a big lump of calcium and transports that calcium into the shell when he is ready for it. So, vertebrates can do it.

HOLTZER: That does not answer my question. Has anyone taken a piece of bone or several of them and grafted them into four or five hunks of muscle to actually trace the haversian system with time?

HEANEY: The haversian systems where?

HOLTZER: In the grafted bone. You posed a problem. I am trying to set up an experiment. If somebody has done it, I would be delighted to know about it. You take a dozen pieces of bone and put



them in a dozen different muscular sites. Then you check those, perhaps 6 months later. What are the haversian system patterns?

URIST: The bone would die.

HEANEY: The bone graft dies initially, but it is invaded then by host cells which ultimately absorb it.

URIST: We are going to discuss that at the next session.



## CELLULAR DIFFERENTIATION IN BONE

**Discussion Leader:**

DR. JOHN J. PRITCHARD

PRITCHARD: Ladies and gentlemen, we shall be talking this morning about "Cellular Differentiation in Bone." The first difficulty is the lack of precision in this word "differentiation." There are several meanings, and quite possibly we shall be thinking about differentiation in different ways at different times. You may have other meanings that I have not thought of, but perhaps for the moment I may be allowed to tell you how I think the word "differentiation" can be parsed and analyzed.

First, the word "differentiation" can apply, and often is applied, to a population of cells. Secondly, the word "differentiation" can apply to some individual cell within a population. It depends on whether our attention is focused on the whole population or on one particular element in that population.

Then again, we can focus on static or instantaneous aspects of differentiation, the idea of differentiation being, like beauty, in the eye of the beholder. The beholder sees a picture; he sees it made up of differing elements and tries to differentiate, delineate, and clarify the elements into species, to which he gives names. This is purely a static analysis; time does not enter into it. You could all die suddenly, but you could still be differentiated by your appearances and classified into groups, and that is normally what the histologist does. He may think he is doing something more dynamic, but really he is analyzing a dead, fixed picture.

The "static" man is simply concerned with differences within a population at a given point in time. The dynamic aspect of differentiation is concerned with change, but even here there is more than one meaning behind the idea of change. There is the idea of the homogeneous becoming heterogeneous, with differences arising and increasing as in the development of an embryo. There is also the idea of the transformation of one mode into another mode without it necessarily being part of an ontologic process.

In these transformations there are those we consider irreversible, sometimes called true differentiation, and those changes which we consider to be reversible, often referred to as "modulation."

Under the heading of "differentiation," therefore, we have population studies, individual cell studies, static studies, dynamic studies. Cellular differentiation in bone thus implies at least four kinds of activity.

FREMONT-SMITH: "Irreversible" means not yet known to be reversible.

PRITCHARD: That is true. It also means that the change is carried on to the next generation of cells.

FREMONT-SMITH: The inheritance of an acquired characteristic.

PRITCHARD: Yes; from one cell to another, not from one organism to another. Some of you may want to add to this analysis of what we mean by "differentiation." In the meantime, I would like to add one or two more guidelines to the discussion. Suppose we begin with the concept of a bone-cell population. These populations are not the same everywhere. There is a great variation in the local populations in different parts of a bone, in different bones, at different ages, in different species, in different functional states. So, we have variations in populations.

There are also variations within a population. This takes into account the heterogeneity within a given population. We are looking eventually for differences between cells. We have to be careful about the criteria we use for differentiating cells, and we have to spend a little time, I think, on nomenclature. What are we going to call those cells once we differentiate them out of the population?

These questions belong to the static level of histologic study. Dynamic studies may be summed up as activities of the population of bone cells. This covers a multitude of topics, and we obviously cannot discuss them all. However, I want to give a fairly complete frame of reference and then select some of the more important issues; you can introduce other important ones.

Under activities of a population, we must not forget the movements of cells, which are sometimes ignored. There are respiratory and secretory activities. There is mitosis, and death of cells. There is the recruitment of cells to a population, and there is the emigration of cells from a population. Finally there is differentiation.

FREMONT-SMITH: You start with a population of bone cells. How did they get that way?

PRITCHARD: You mean, do we get cells from outside?

FREMONT-SMITH: There is an ontogeny of these cells—

PRITCHARD: An ontogeny which consists of mitosis followed by migration, differentiation, modulation, transformation, functional activity; in other words, the population has a lively history which can

be traced back to embryonic beginnings at an ossification center, and perhaps even earlier. Moreover, these activities are under control.

In principle, I think there are three kinds of control that can be exerted over the activities of the population. First, there is control from outside the population, directly issuing from sources external or extrinsic to the population. One thinks of hormones, mechanical factors, toxic substances—anything that can get at a population from outside.

FREMONT-SMITH: Do you consider induction as part of that?

PRITCHARD: Induction is sometimes controlled from outside, sometimes from inside.

FREMONT-SMITH: You are starting as if we already had a population of bone cells.

PRITCHARD: We will go back and prove that there is a population later on. But assume that there is a large population of cells and that these cells are active in some of the ways I have mentioned; then we could have influences on the activity of a population arising from outside the population. We could also have mutual influences of the cells upon one another within the population.

We also have influences or determinants which come from the cell itself, the DNA in one cell perhaps being in a different state from the DNA in another cell. Therefore, there are controls extrinsic to the population and controls intrinsic to the cell.

Then, there are some final considerations: How do these controls work? Do controls affect the population by affecting the mitotic rate of some particular stem cell? Do they affect the secretory activity of some already modulated cell? Do they influence membrane functions in some of the cells? Or do these controls work at the DNA-RNA synthesis level? Just what kinds of controls and regulations are present, and how do they work?

These are my four major headings: the static attributes of the population, the activities of the population, the controls, and the mechanisms of control. Some key problems center around the criteria for identifying cell types and giving them names. The most important problem is tracing individual cell lineages through serial mitoses and isolation.

The most pressing problems, however, concern the controlling mechanisms—and we tend to think immediately of induction and hormone action. I would like to add to these the action of the vascular system on the population; there are also mechanical and nutritional, even nervous, influences to be considered. In the last analysis, all these influences may well have a final common path through the cell nucleus; at present we are at the stage of cataloging the factors and their visible and chemical effects on the population.

Returning to the concept of bone-cell populations, I would like to emphasize the continuity of the populations. We do not very often see a bone as a whole or think about its cell population in toto. In histologic preparations of small bones from young animals, one does get the idea of a continuum of cells within subpopulations in the periosteum, the bone marrow, the cortex, the metaphyses, and the epiphyses. When we discuss the characteristics of these subpopulations we would do well to remember that they are part of a continuum which starts from the periosteum, goes through the bone cortex, and on up and down the marrow into the metaphyses.

Let us consider bone from the point of view of population. A young bone has a fairly open cortex—that is, the periosteum—with part of its bone-cell population, the periosteum, in place. This is continuous with the bone-cell population, working through the interstices of the cortex. Then there is the population of the bone marrow through and including the special population of the endosteum at the surface of the bone marrow. Then we have the population going up into the resorption spaces, and then we have an independent population of the bone cells in the epiphysis. While this is an independent population, all the rest are really forming a continuum; there is no very great difference, in many cases, between the periosteum population in the cortex, the population within the marrow, and the population in the metaphysis. I think we were trying to differentiate between metaphyseal bone, cortical bone, and cancellous bone as if each were an entirely different species of bone. I think, in reality, if we trace their development we could see how the appearance of the bone gradually hardens and crystallizes out from a fairly basic unified pattern.

In any section of bony tissue taken from a young animal, one is liable to be confronted with a bewildering variety of cell types. There are plump osteoblasts and multinucleated osteoclasts on bone surfaces, and osteocytes of different kinds imprisoned within the bone matrix. There are “fibroblasts” in the outer layers of the periosteum and in the connective tissue around the larger blood vessels of the bone marrow. There are endothelial cells of blood vessels, leukocytes and erythrocytes in all stages of maturation, and fat cells. In the chicken bones I have been studying there are masses of nucleated erythrocytes in the blood vessels, which adds to the confusion. In addition to all this, there are certain nondescript, unspecialized-looking cells to be seen almost everywhere; these cells do not fall into any of these categories. They are present in the depths of the periosteum, around the fine vascular channels of cortical bone, in the spaces of cancellous bone, lining the medullary surface of the cortex, and scattered throughout the bone marrow. They have been given many names, and they have long been regarded as a reserve of uncommitted cells from which new osteoblasts

are recruited during growth and repair. Their status has been made much clearer since the introduction of tritiated thymidine as a nuclear label. It is now realized that these reserve cells can multiply and differentiate into a variety of specialized cell types including osteoblasts, osteoclasts, and chondroblasts.

In mature bone the case is quite different. Plump osteoblasts and osteoclasts are found only occasionally, most bone surfaces are covered with exceedingly flat cells, and the reserve cells mentioned are very difficult to identify. Yet if mature bone is stimulated by injury, for example, its cell population rapidly takes on the appearance it had in its youth. The flat cells rapidly fatten into osteoblasts, and near them reserve cells become conspicuous. Osteoclasts also soon make their appearance. It would seem that the cell population can go into hibernation, and then be awakened.

If we grant that bone contains a ubiquitous population of multipotential reserve cells, then the consequences of stimulating them to divide and to differentiate will be far reaching. Indeed, the obvious method of regulating bone activity would be exerting control over the multiplication and differentiation of these reserve cells. I should expect that much of our discussion will center around these cells and the factors which regulate their activity. But first perhaps we should agree on a generic name for them.

URIST: Would you call them mesenchymal cells?

PRITCHARD: I do not think that the name is as important as knowing that they are there. I think it is more important to delineate them by definite properties—what they can do, what they cannot do, how they stain, and so forth.

URIST: If the animal had one injection of tritiated thymidine, where would the label be located?

PRITCHARD: I suspect it would go into some members of the reserve-cell population in the first few hours.

URIST: The  $^3\text{H}$ -thymidine would not go into the endothelial cells in any great amount.

PRITCHARD: No; because they are not about to divide.

URIST: The next layer, the nondividing cells, would not utilize a large amount of  $^3\text{H}$ -thymidine. Only the population of cells, the progenitors, preparing to divide rapidly and in large numbers would be expected to be labeled with  $^3\text{H}$ -thymidine.

PRITCHARD: But you would not know it was about to divide unless it took up thymidine and told you so. Otherwise, you probably would not notice it.

BÉLANGER: I think we know what our chances are for seeing the thymidine go to one area or another by the amount of mitotic figures which we can observe in a normal preparation.

PRITCHARD: Where does it go to? You mean in relation to the bone as a whole?

BÉLANGER: Yes. I am trying to get an answer for Dr. Urist. The use of thymidine is a very sophisticated approach to this kind of thing. Before thymidine was available it was possible to predict where it would go proportionately by the amount of mitotic figures that we could observe, particularly after colchicine, a very simple technique that anyone can do.

PRITCHARD: This comes under the question of the criteria we are going to use to differentiate different types or modulations of cells in a population. Could we keep to static considerations for a while, and deal with dynamic aspects later?

When you administer thymidine and label a cell, that presumably is a static study. You do not actually see the cell dividing; you merely have evidence that it is about to divide or that it has divided.

On this basis, Dr. Bélanger, what types of cell would you recognize in a bone population?

BÉLANGER: Do you mean in relation to thymidine?

PRITCHARD: Yes; in relation to thymidine. You can divide the population in many ways: in relation to phosphatase, in relation to glycogen, in relation to size and shape of cells. However, in relation to thymidine, what cells would you recognize?

BÉLANGER: I think we should ask Dr. Young, who has firsthand information on this sort of thing. I think we agree on some aspects about that. Some people do not agree, I believe, on the actual names which are given to these cells, but we all agree that they are what you call reserve material, what Cronkite et al. (ref. 108) call primitive cells, what Young calls osteoprogenitors (ref. 109), and what embryologists like to call mesenchymal cells; they are all the same. These are the cells in which you would expect most of the thymidine to be located—in all those areas, including the endothelial wall where there is a very rapid renewal of the cell population from other endothelial cells.

PRITCHARD: Do they remain endothelial?

BÉLANGER: I do not know, but certainly the endothelium renews itself in great part, except wherever new vessels appear from the mesenchyme from other cells in the wall.

PRITCHARD: Dr. Young, would you talk about the distribution of tritiated thymidine in a bone-cell population, without perhaps at this stage going into family histories and so on, but sticking to what you actually observe when you use thymidine?

YOUNG: My experience has been that one can distinguish at least four functional states of the bone cell. These states would include the three classic cell types: osteoblasts, osteoclasts, and osteocytes.



In addition, there is a pale-staining, rather spindle-shaped, cell which so far we have been hesitant to name. I call this cell the osteoprogenitor. It utilizes tritiated thymidine in synthesizing DNA prior to cell division and serves as a source of new bone cells.

The classic cell types are, generally speaking, incapable of reproducing themselves. However, the bone cell is capable of assuming a specialization in which it can divide, and it is in this state that it takes up thymidine.

While we are on the static aspect, I would like to show some figures which indicate that although (I will develop this later if time permits) the three classic cell types are all ultimately derived from cells produced through mitosis of the osteoprogenitor, they are nevertheless functionally different.

PRITCHARD: Is that static? I would have thought that was a dynamic problem.

YOUNG: I have proof for this statement, but have only mentioned it now as a background for the figures I will show.

If we examine, by autoradiography, the specialized cells in bone  $\frac{1}{2}$  hour after injecting tritiated glycine, we immediately see that these cells are functionally different (ref. 110). Figure 60(a) is an osteoclast which has utilized, relatively speaking, practically none of the injected glycine; whereas the osteoblasts in the very immediate vicinity, having access to the same tissue fluid, have concentrated the glycine in their cytoplasm in the act of protein synthesis. Glycine shows the greatest contrast between these two cell types because it is the most common amino acid in collagen, which is, of course, what these osteoblasts are largely synthesizing.

PECK: What is the evidence that glycine is in protein? There are many functions for amino acids in a cell besides incorporation in protein.

YOUNG: It can be shown biochemically that if one supplied these cells with tritiated glycine or  $^{14}\text{C}$ -glycine, the radioactivity can shortly thereafter be recovered in collagen; but autoradiographers using this sort of technique have to accept the fact that practically all low molecular weight compounds are washed out in preparation of the sections. The result is that we are looking, in a sense, at a nucleoprotein carbohydrate residue of what used to be there.

Within 4 hours, most of this labeled protein is released from the cells (fig. 60(b)). The osteoblasts are now essentially devoid of radioactivity, and they have laid it down on the surface of the matrix.

Osteocytes, which are, after all, just trapped osteoblasts, also use tritiated glycine (fig. 60(c)); at least the new ones do, such as we saw yesterday in the elegant micrographs presented by Dr. Robinson.

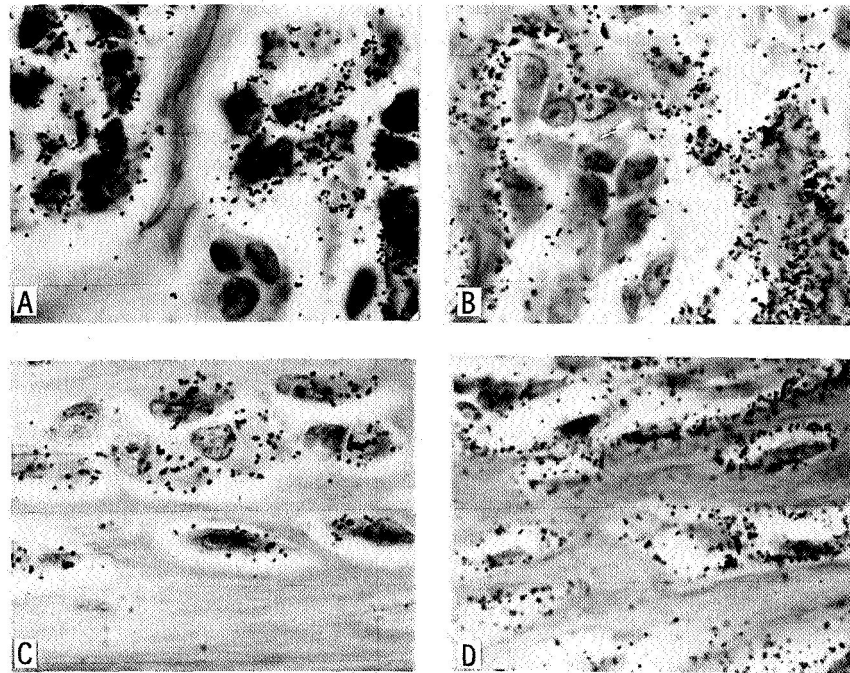


FIGURE 60. Photomicrographs of sections from tibial metaphysis of 1-week-old rats, sacrificed 30 minutes after injection of  $^3\text{H}$ -glycine. Autoradiograph, PAS-hematoxylin stain. 900  $\times$ .

(a) Osteoblasts have concentrated large amounts of the labeled amino acid. Note that the osteoclast (below, center) has incorporated very little of the radioactive protein precursor.

(b) The osteoblasts have deposited radioactive protein on the surfaces of the adjacent bone trabeculae.

(c) Newly formed osteocytes (above) are actively synthesizing protein. Older osteocytes (below) are relatively inactive in this regard.

(d) Newly formed osteocytes have deposited the radioactive protein on the surface of their lacunar walls.

These cells are also synthesizing bone matrix. The older osteocytes, which are smaller and in smaller lacunae, use little or no glycine.

Within 4 hours, the newly formed osteocytes lay down the radioactive matrix on the surface of their lacunae, which they are remodeling into smaller, more almond-shaped cavities (fig. 60(d)).

BÉLANGER: Is this a rat, Dr. Young, or a mouse?

YOUNG: This is rat bone. I have been talking here about glycine. We have also looked at many additional amino acids (ref. 111). From these figures, one might draw the conclusion that the osteoclasts and osteoblasts were doing the same thing, but at different rates. However, if we try a large series of precursors, we will find that the labeling

relationships between osteoclasts and osteoblasts differ. They are handling each precursor according to their own preference.

With some precursors, such as proline, there is still a high ratio of labeling in osteoblasts as compared with osteoclasts. In others, such as alanine (fig. 61(a)), the distinction is perhaps not quite so great.

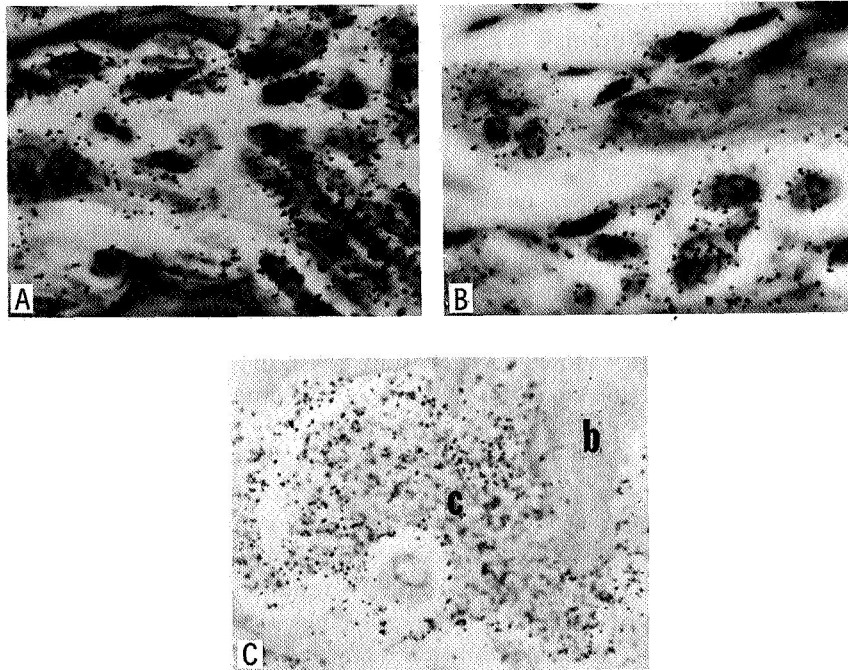


FIGURE 61. Photomicrographs of sections of tibial metaphysis of rats after injection of tritiated compounds. Autoradiograph, PAS-hematoxylin stain. 900  $\times$ .

(a) One-week-old rat, sacrificed 30 minutes after injection of  $^3\text{H}$ -alanine. Difference in labeling between osteoclast (left) and the osteoblasts is not as marked as with glycine and proline.

(b) One-week-old rat, sacrificed 30 minutes after injection of  $^3\text{H}$ -histidine. Difference in labeling between osteoclast (left) and osteoblasts is even less.

(c) Eight-week-old rat, sacrificed 1 hour after injection of  $^3\text{H}$ -taurine. Large, heavily labeled osteoclast (c) on surface of unlabeled bone matrix (b) appears to be engulfing an unlabeled cell (below, center). Glutaraldehyde fixation.

With histidine (fig. 61(b)), the labeling in osteoblasts and osteoclasts is fairly comparable, although on a per nucleus basis it is still higher in osteoblasts.

In some cases, such as this unusual precursor (fig. 61(c)), we find that the osteoclasts are very strongly radioactive, and the osteoblasts are relatively less heavily labeled.

This label is taurine; it is not an amino acid. It is believed to be a waste product, a breakdown product of the sulfur-containing amino acids. It is actually the first precursor that I have found which appears to be preferentially concentrated in osteoclasts.

I think that although we are able to distinguish these cells morphologically, these differences in appearance reflect underlying differences in the metabolic organization of the different functional states of the bone cell.

NICHOLS: Dr. Young, is it not true that the osteoblasts may be active in this sense in some areas of the bone, and in other ways not so active? Can you really use amino acid uptake as a reliable criterion for identification?

YOUNG: I have never observed a cell which was a full-blown osteoblast with well-developed endoplasmic reticulum, Golgi complex, and so on, that did not avidly concentrate protein precursors. One expects to find osteoblasts that are beginning to respecialize, perhaps as osteoprogenitors, or osteoprogenitors that are beginning to specialize as osteoblasts. These will not show the full response, the highest rate of bone formation. However, generally speaking, any cell which is histologically recognized as an osteoblast will, upon autoradiographic analysis, prove to be synthesizing bone matrix.

HOWELL: How old are the animals that you studied?

YOUNG: These are young animals. In old animals in a region of quiescence in bone, the osteoblasts are not fully developed; they tend to be flattened. They will show some uptake because they are indeed alive, their enzymes are turning over and so on; but here again the morphologic picture, for those who are not using autoradiography, can serve as a fairly good guide to the activity of the cell.

BÉLANGER: Is there a parallel between the cells that pick up thymidine and those that pick up the amino acids?

YOUNG: All cells that are alive are going to use some of the amino acids. Those that have reorganized their metabolic machinery in order to synthesize collagen are nondividing. Dr. Holtzer would agree with me, I think, that the cells which are highly specialized for some specific function are generally not dividing cells.

NICHOLS: A couple of years ago Dr. Owen made some autoradiographs of bits of pig bone which I had incubated with tritiated proline. We observed quite active uptake in the osteoblasts in some areas and in other areas practically none. We also observed labeling of osteocytes similar to that with glycine. Dr. Owen, is my memory correct?

OWEN: Yes. However, I would like to make one point. In very young animals (about 1 week old), such as Dr. Young and I have used, all the osteoblasts are actively synthesizing collagen. In older

animals only selected regions of osteoblasts synthesize collagen; other regions are quiescent. Dr. Nichols' material consisted of bone chips from older animals.

NICHOLS: All right, but what I am trying to get at is that an osteoblast can be considered an osteoblast, at least by a biochemist, and still not be making much collagen at the time. I think that this is an important point because I have the notion that differentiation can occur without the cell necessarily having to perform the function for which it is now differentiated. In other words, a runner does not necessarily have to be running all the time to be recognized as a runner. He can occasionally stop.

RAISZ: Were those precursors given *in vivo* or *in vitro*?

NICHOLS: *In vitro*.

RAISZ: I would object to the interpretation on the ground that the function of some of the cells may have altered since they were removed from the animal.

TALMAGE: I am sure there are differences in the rate in which various osteoblasts take up protein and synthesize collagen. For example, it has been demonstrated in the rat that osteoblasts in the metaphyseal region of the femur turn over radioprolin at a much faster rate than do osteoblasts in the shaft of the same bone.

NICHOLS: We can demonstrate very clearly that proline went through the osteoblasts in the metaphysis at a much faster rate than it did through the osteoblasts in the cortex of a rat bone. After one-half hour a fair amount of metaphysis had gone into collagen whereas in the diaphysis, 96 percent was still left in the cell. We followed through to 4 hours and were able to draw a curve on the rate in which osteoblasts in different parts of the bone were synthesizing proteins.

PRITCHARD: As a matter of fact, we are trying to get to the criteria for distinguishing species of cell in the population. These different cell types are going to vary in their activity from time to time. Using the criterion of labeled amino acid uptake, there appear to be differences between the kinds of cell which morphologically we term osteoblasts, osteoclasts, and marrow cells.

Metabolic criteria are important, but we must not forget that there are other ways of distinguishing the elements of this population. As I have indicated, shape, size, and gross internal structure were all that the classic morphologist had to go on for a number of years. When he saw a cell with long, thin processes, with a large nucleus at one end, an enormous negative Golgi area in the middle, a basophilic cytoplasm full of mitochondria, and when that cell was sitting on a bone surface, he called it an active osteoblast.

When he saw a much larger cell with many nuclei, a large number of mitochondria, and residing in an excavation on the bone surface,

he called it an osteoclast. He forgot to talk about or only mentioned in passing, as a rule, the other cells present, because they did not have these very distinctive morphologic features.

Then there are staining reactions. The cytoplasm may be either eosinophilic or basophilic, or a mixture of the two.

Another criterion, of course, is enzyme activity. There seems to be no limit to histochemical criteria. One cell type may have very high alkaline-phosphatase activity, whereas another cell type may have a very high acid-phosphatase activity. Succinic dehydrogenase activity may be high in one type of cell, and in another type there may be low isocitric dehydrogenase activity and so on.

We have not yet mentioned tritiated thymidine, which is mainly used to determine family relationships within a dividing, differentiating population. But thymidine is also a static criterion in that we can distinguish certain cells that take up thymidine immediately from those that do not. I am sure there are many other physical and chemical criteria waiting to be tried. We certainly have enough criteria. Are we in a position to classify the cell types and agree on names for them? What shall we call the cell that lies on a bone surface looking like an osteoblast that has been flattened? It still has a nucleus at one end, it still has the big negative Golgi image, and it still is basophilic; but it is as flat as a pancake. This cell type is rarely mentioned.

Nevertheless it is ubiquitous and, moreover, I have seen many transitions between this type of cell and the plump classic osteoblast. I would like to suggest that the flat cell is a resting osteoblast, and the plump cell is an active osteoblast. It seems that when an osteoblast has stopped being very active, it may not necessarily go back to a progenitor stage but may just go back to a resting stage, while retaining its potential as a working cell.

BÉLANGER: It is a very important point, Dr. Pritchard, if I may say so, that not all osteoblasts become osteocytes. Some become resting osteoblasts. I do not know now whether I agree with Dr. Young that they may go back to being mesenchymal cells or young cells, but some active osteoblasts, a fairly large number—depending on where we are looking in the bone and depending on the actual growth potential of the site—will go into the resting state; we can follow the thymidine through time into these or into osteocytes. In other words, the number of osteocytes does not account for the number of osteoblasts which showed the thymidine previous to that stage.

SAXÉN: Could you define the resting stage?

BÉLANGER: This is the particular stage that Dr. Pritchard discussed.

PRITCHARD: Most bone surfaces in the adult are covered with a pavement of flat cells. These cells have certain osteoblastic features. Their long processes go into the bone, the nucleus is at one end, and

there is a large central Golgi zone; but they do not have the amount of cytoplasm or the number of organelles. They are literally at rest, but there is no evidence that they are engaged in bone formation or resorption. These are the cells that we need to name. I call them resting osteoblasts.

NICHOLS: Does anybody have an electron micrograph of one?

PRITCHARD: Dr. Robinson showed them.

OWEN: Our studies of the kinetics of cell differentiation on the periosteal surfaces of young rabbit femur may be of interest at this point. We have measured the rate at which preosteoblasts—perhaps I should not use that term—

FREMONT-SMITH: Why not?

OWEN: I mean the rate at which the precursor cells of the osteoblasts differentiate to become osteoblasts, and then the rate at which the osteoblasts go on to become either osteocytes or osteoblasts living in haversian canals. We found no evidence of any cell death in this system. All osteoblasts originally on the surface eventually became either osteoblasts in haversian canals or osteocytes within the matrix, about 60 percent and 40 percent, respectively, in the two categories (ref. 112).

FREMONT-SMITH: No resting cells?

OWEN: The osteoblasts in haversian canals, especially those deep within bone, are resting osteoblasts.

PRITCHARD: Dr. Owen, would you tell us what your criteria were? I have given my criteria for the population of resting osteoblasts. What would your criteria be?

OWEN: In our particular study it was not necessary to define resting osteoblasts. However, I am more or less in agreement with your definition. In our material, haversian canals that are deep within the bone have osteoblasts on their surface which I would describe as resting. The cells are flattened against the surfaces of the lumen and do not show detectable labeling with tritiated glycine.

PRITCHARD: In other words, location, rather than any specific morphologic feature.

OWEN: Yes; in this particular study the main criterion was location.

MCLEAN: What about the alkaline phosphatase?

OWEN: I would like, at some time, to put in a plea for the term "preosteoblast," because I think there may be several stages included in the osteoprogenitor stage of the cell. Balogh and Hajek (ref. 113) found different histochemical staining reactions in osteoprogenitor cells in different situations. In their studies, what they describe as the osteoprogenitor cells of the periosteum show a moderate staining activity for isocitric dehydrogenase and glucose-6-phosphate dehydrogenase, whereas these enzymes were not demonstrable in the

osteoprogenitor cells of fracture callus. They also describe, in fracture callus studies, mononucleated cells showing succinic dehydrogenase reactions; the enzyme specific for osteoclasts in bone. They suggest that these mononuclear cells may be precursors of the multinucleated osteoclasts—might I suggest the term “preosteoclast.” Walker (ref. 114) also described mononuclear or binuclear cells with strong succinic dehydrogenase activity in bones treated with parathyroid hormone. If the proliferating precursor stage of bone cells—the osteoprogenitor stage (ref. 109)—does consist of several stages, this would fit well with what has been found in other tissues; for example, the well-known multiple stages of proliferating precursor in the blood series. More recently Combs et al. (ref. 115) reported several stages which were histochemically and autoradiographically distinguishable of proliferative precursors in mast cells.

URIST: Does Dr. Owen propose that a gradation of changes in cell metabolic reactions could occur in the course of mitotic division?

PRITCHARD: She did not really say that.

URIST: I will restate the question. There is a series of mitotic divisions, and there is an arrangement of cells in layers. The layers consist of perivascular connective tissue cells, mesenchymal cells, and connective tissue cells closest to the bone, which are preosteoblasts. In my mind this raises the question of whether another criterion, the one Dr. Nichols mentioned, that of ultrastructure, can rescue us from the dilemma of morphology under the light microscope.

Can Dr. Robinson and his associates, who are familiar with these cells in the electron microscope, distinguish between a perivascular connective tissue cell in a muscle and a perivascular connective tissue cell in a bone? I think that is what I am searching for in order to identify a cell by its potential for function.

PRITCHARD: We are trying to arrive at some objective, incontrovertible criteria for saying, “This is an X cell; this is a Z cell; this is a dead cell.”

URIST: Will the criterion of ultrastructure contribute something in addition?

PRITCHARD: I think it will help, but we need evidence from many different sources; we will have to say where the different criteria overlap and congeal into definitive species, like separating out the animals of an animal population. One criterion does not, in general, enable you to distinguish one species from another. You have to integrate a lot of criteria before you can say, “This is one species, quite distinct from that species.”

URIST: Dr. Robinson, can you distinguish between a preosteoblast and a premuscle cell? The two can be found in adjacent areas in every section of bone tissue.



ROBINSON: No.

URIST: There is no ultrastructural characteristic?

ROBINSON: I am not saying there is no ultrastructural characteristic. It is just that I have not studied the difference between a preosteoblast and a premuscle cell.

URIST: Let us say a preosteoblast and a prehematocytoblast. They are all right there.

ROBINSON: Well, I think that one of the things that has fascinated us in the study of the haversian canal is that there is really a three-cell layer, even in the young canal; there is the endothelial cell, the cell that lies under the endothelial cell, and the cell that lies next to the bone. The cell that lies between the endothelial cell and the bone cell has some characteristics of both, if you want to speak about morphology of fine structure of the cytoplasm.

This is described by Cooper et al. (ref. 25), but we are not sure what these slight resemblances mean because, after all, the morphology may have something to do with the function of the cell at the moment.

What I am interested in now is where these osteoblasts can possibly come from in many sites in bone; for instance, up in the area where new bone is forming on trabeculae of calcified cartilage. Trueta (ref. 116) has pointed out that the only cell around seems to be the endothelial cell, and he, I think, stated quite definitely that the endothelial cell became an osteoblast.

PRITCHARD: Many people would not agree that there are no other cells around, that the endothelial cell is the only candidate. They would point to the mesenchymal population around the blood vessels.

ROBINSON: Well, we felt quite differently than Trueta did. We felt that the endothelial cell might give rise to the precursor of the osteoblast, but we were in doubt as to the origin of the endothelial cell itself. Could it be one of the functional forms of a monocyte?

I would like to ask Dr. Owen about statements in her paper in which it was mentioned that thymidine was picked up by the endothelial cells, by the progenitor of the osteoblast, also once in a while by the osteoblast, and even by the surface osteocytes, cells which I do not think would be expected to divide. I think Dr. Owen referred to Pelc in this regard.

OWEN: I think you are referring to the paper (ref. 112) where we were studying the growing surface of young rabbit femur. The osteoblasts are on the bone surface; the precursors of the osteoblasts, termed the "preosteoblasts," are behind the osteoblasts. These latter cells were the main region of thymidine uptake at short times after injection. They may well have included endothelial cells; we did not try to distinguish these. We never found early thymidine labeling of osteocytes. Labeled osteocytes were found, but at later times, due to the fact that

the cells had taken up the thymidine at an earlier stage, probably as preosteoblasts.

BÉLANGER: Certainly, migration.

OWEN: Migration, yes. Perhaps you are referring to a second paper; there were two papers. Are you referring to the first or second?

ROBINSON: I am referring to the paper by Owen and MacPherson (ref. 117).

OWEN: Yes; that was the second paper. As you know, incorporation of thymidine into DNA occurs during the process of doubling of the cell's DNA in preparation for cell division. All cells that have taken up thymidine should have divided by about 17 hours after injection. We found that a certain proportion of the cells that took up thymidine did not appear to have divided by one, two, or more days after injection. Our evidence came from grain-count studies where we showed that a proportion of the cells at later times had the original grain count of cells 1 hour after thymidine injection. As yet we have not made any further progress on this matter, and I do not know what its significance is.

PRITCHARD: Does this include the osteoblasts?

OWEN: A small number of osteoblasts do take up thymidine; other people have also reported this. However, this could possibly be explained in terms of our criterion for distinguishing preosteoblasts from osteoblasts. We distinguish in terms of location only so that a labeled osteoblast could in fact be a preosteoblast in the wrong location.

BÉLANGER: Dr. Owen, did you not use the double-labeling method at one time to show a label in the nucleus and a label in the cytoplasm—let us say, protein synthesis in cytoplasm and nuclear labeling from thymidine?

OWEN: I think you are referring to our experiments using tritiated glycine.

BÉLANGER: I think what you did was to show incorporation in the matrix from cell.

OWEN: Yes. Glycine is taken up into collagen; i.e., glycine is first incorporated into the osteoblast and then laid down in matrix collagen. In addition, as Dr. Young showed, there was also some uptake of glycine into young osteocytes. These are the osteocytes near a growing bone surface, and in more recent work I have found that they also take up a little RNA.

YOUNG: RNA precursors?

OWEN: RNA precursors, at a low rate.

YOUNG: I think that the terminology has been perhaps a little obscure here. I feel that we should recognize in bone four major functional states of the bone cell. One of these is a dividing state, which I call the osteoprogenitor, and this osteoprogenitor, the offspring from this

cell, can specialize as either osteoblast or osteoclast. If given the opportunity, I will try to demonstrate that to you. That is why I feel it is misleading to call this dividing cell either a preosteoblast or preosteoclast because it implies that you know, before it has specialized, how it is going to specialize.

Now, if the osteoprogenitor can specialize as an osteoblast, which it can, there must be a stage when it is accumulating the intracellular machinery that will ultimately characterize it as an osteoblast. During this specialization process, which is a matter of a few hours (ref. 109), it will have some of the characteristics of an osteoblast, but will not be a fully developed osteoblast. If we wish to use the term "preosteoblast" for this cell when it is in the process of specializing, I do not see any objection to that. However, I would like to point out that a problem arises if you are observing an osteoblast that is respecializing as an osteoprogenitor. You may call it a preosteoblast when it is actually postosteoblast.

PRITCHARD: This is a big question, reversibility. We must discuss reversibility at some time because it is crucial to the whole problem of cell physiology.

HOLTZER: Can I make a plea that this discussion, which really could have taken place 30 years ago, is still taking place, and—

PRITCHARD: We did not have any tritiated thymidine then.

HOLTZER: That is it. So let us forget the guidelines of 30 years ago and focus on Dr. Young's elegant model. There is a population of cells. Some of them can be identified under the microscope, others cannot. I think some of the questions raised, "Do they all work?" and "How can they be recognized in the process of transforming?" are very provocative, but how can they be approached experimentally?

PRITCHARD: I think we ought to communicate.

HOLTZER: We have an operational definition: At a given time a given cell is engaged in making A, B, and C kinds of molecules. At a later time can that same cell engage in making X, Y, and Z molecules? In brief, let us not worry about names; rather, let us be concerned about the experimental evidence which shows that before, during, or after a given mitosis, a cell synthesizes this or that kind of molecule. So many questions we ask cannot as yet be answered in terms of current methodologies.

PRITCHARD: All right. Let us leave the terminology at this stage by agreeing that underneath the fiber layer there is a progenitor layer. Then there is the next layer which the problem is really about; what are we going to call this layer, before we get down to the cells that everybody agrees on? What are we going to call these intermediate cells which do not multiply as fast as those of the other layer. The term

mostly used is "preosteoblast," but should there not be preosteoclasts and prechondroblasts? What should we call the intermediate cells?

OWEN: I have pointed out some differences that have been demonstrated within osteoprogenitor cells, but I do not want to emphasize them. For the moment I think Dr. Young's scheme is an excellent working basis: osteoprogenitor cells and the functional cells of bone including osteoblasts, osteoclasts, osteocytes, chondrocytes, and so forth. However, I am of the opinion that differentiation is probably not a one-step process. It is a gradual accumulation of characteristics until the fully differentiated cell is achieved. Eventually we will be able to distinguish the different states, which I am certain exist, among osteoprogenitor cells; but for the moment I propose that we stick to Dr. Young's scheme.

RAISZ: I would like one point clarified. Is cell division required to go from one of these cell types to another? This is the implication now, but from things said earlier, I did not think it was true.

YOUNG: Cell division is required to make new cells.

RAISZ: That is helpful. It is not necessary to divide in order to go from one to the other?

YOUNG: No.

OWEN: That is another question, another problem.

PRITCHARD: Please bear with me for a moment. This question of the relationship of these cells to each other and the criteria for differentiating them is not purely academic. Many advances in bone physiology depend upon getting the right answers, and we also have to establish a firm base for genetic studies. I should like to stress that Dr. Young is not the only man in the field so far as these relationships are concerned. Frost (ref. 118) has a scheme in which the cells go in one direction and all die at the other end, he has no reversibility in his system. Other people have modulation effects, side effects, one cell turning into another. Dr. Young's scheme is a reversible scheme. These differences are significant and important. I would like to know what the evidence is before we take up the more dynamic aspects.

BÉLANGER: I would like to object to the statement just made by Dr. Owen. I think the term "osteoprogenitor" was proposed by Dr. Young to apply to normal events only. If you have a fracture, for instance, you can see that these cells which are supposed to be under normal conditions, just progressively becoming bone cells, will now start making cartilage in the same site. Therefore I do not think that there is, at this moment, a very fixed destiny of the cell. This cell can divide. This cell is an ancestor of some kind; but depending on the local or general conditions of the organism, the cell could just as well turn into a cartilage cell, into a blood cell, or into all sorts of things. So I think that the old term "mesenchymal cell" or the term "stem

cell," such as is used by Cronkite et al. (ref. 108) in their excellent work, does not commit that cell to any specific destiny.

NICHOLS: I think the thing we really want to know now is whether one can identify these cells on a functional basis; and if so, how?

BÉLANGER: It is for Dr. Nichols' benefit, mainly, that I would like to answer that question and stress the point about the resting, or let us say "lazy" osteoblasts in relation to active ones. Figure 62 shows a "lazy" one seen at low power. We can see that it has already been growing processes next to the surface bone, but it has a fairly well-developed endoplasmic reticulum, and a few processes on the opposite side. But the main character of this cell, which distinguishes it from an active osteoblast, is the lack of development of the Golgi complex. This cell has practically no Golgi apparatus. If one can distinguish where, in time, this cell belongs in the lineage of migration of cells after

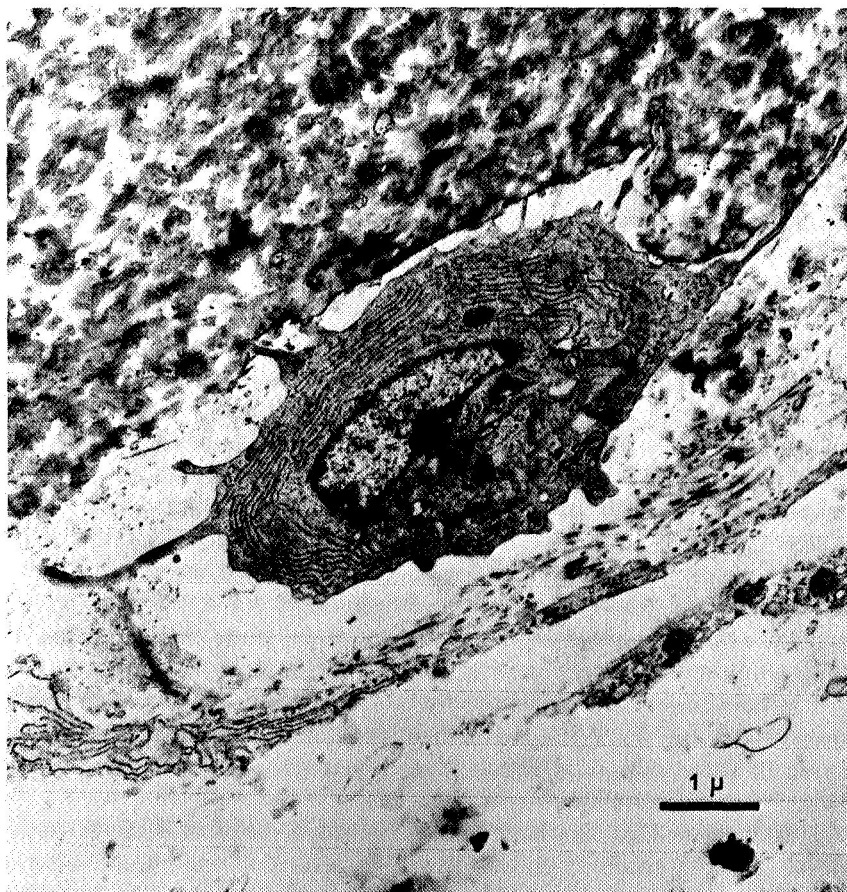


FIGURE 62. Osteoblast from tibial diaphysis of 11-day chick embryo.

it has picked up thymidine, this cell is not an active osteoblast. For some reason it is not manufacturing protein in large amounts, or other constituents of bone. It is a resting cell, but it is already a highly differentiated cell.

YOUNG: How do you know it is not synthesizing protein?

BÉLANGER: I do not say it is not. It does not show that it is active at the same rate as the large osteoblasts Dr. Pritchard has described in which one can see a very well developed Golgi apparatus and an endoplasmic reticulum with large cisternae. In this cell there are no cisternae and no Golgi complexes.

NICHOLS: Dr. Young, when you see osteoprogenitor cells, do they take up precursors, such as glycine or proline?

YOUNG: You show only a tiny slice of one cell. I am not convinced there might not be a Golgi complex in the next section.

BÉLANGER: This is a low-power thing, and I realize that you have not seen the whole cell; but Dr. Nichols asked for a distinction. The only distinction I could suggest would be that the Golgi net is small at the endoplasmic reticulum.

NICHOLS: I would like to comment on this discussion because I think it is very important for several reasons. I submit that morphologic criteria, such as presence or absence of rough endoplasmic reticulum, are not sound evidence in assessing the activity of the cell. The mere presence of equipment does not mean that it is being used.

The time required for a cell to get ready to make protein under a stimulus may well depend upon how much of the needed equipment it has; perhaps this is differentiation—at least in one sense. The point is that there can be various kinds of cells in various stages of activity depending on a whole series of stimuli. While it may be quite different to equate a given cell to a given job, this is going to be very important if we are going to understand (1) how the tissue is formed, (2) how it is taken away, and (3) what goes wrong with the system when we get sick.

PRITCHARD: Consider the flattened cells on the surfaces of adult bone. If you fracture the bone, these cells have become plump, typical osteoblasts and have started to make bone matrix within 12 hours. So they can get their machinery in top gear in 12 hours.

FREMONT-SMITH: Are there any tissue-culture studies which would throw light on this? Because then you can get moving pictures of some of these cells and watch them when they are doing it. Is any material on this available?

URIST: Yes; that has been done. When a piece of bone is put in tissue culture, there is an outgrowth of cells that are spindle-shaped connective tissue cells, that look like mesenchymal cells, but they exhibit a different capacity for development.

FREMONT-SMITH: Do you know where they come from?

URIST: We assume they come from osteoprogenitor cells. If you use a cataract knife to remove a bone explant from tissue culture and transplant the outgrowth back into a living rat, the cultured cells will differentiate into osteoblasts. Cells that look like undifferentiated connective tissue cells can be different insofar as they can exhibit osteogenic potency. If one looked at the spindle-shaped cell of the outgrowth under the electron microscope I do not know what one would see; I do not know anyone who has done this experiment to investigate the ultrastructure of the cells of the outgrowth.

FREMONT-SMITH: Some of the moving pictures of tissue-culture cells in the central nervous system, for instance, have changed our views so strikingly that I thought maybe this would answer some of the questions Dr. Nichols raised. I thought maybe we were using the wrong criteria for making decisions as to what a cell can do or has been doing.

NICHOLS: I am not acquainted with those observations so I cannot comment, but certainly one of the problems with tissue culture is dedifferentiation, which seems to be a particular plague of cultures of fibroblasts and connective tissue cells at large.

URIST: This is correct. Dedifferentiation does occur, after two and three generations of cell culture. Eventually the cells can no longer make bone. The more recently the cell has had contact with bone, the more likely it is to be able to make bone when it is grown out on tissue culture and returned to an intact animal.

FREMONT-SMITH: You mean only after the cells were transplanted back?

URIST: Yes; after the cell has been away from the animal and away from the bone for a long time, after the third and fourth generations have been cultured and recultured, the cells of the outgrowth lose their potency to differentiate into bone after transplantation.

PRITCHARD: Müller (ref. 119), one of the great pioneers of tissue culture, subcultured osteoblasts about 19 times, if I remember correctly, and yet they kept their potency. This does not agree with what you say. The cell may look different, but it keeps its potency.

NICHOLS: I submit that the cell does not even have to be near bone. I remember some experiments by Dr. Huggins in 1931, in which he transplanted bladder epithelium and got bone (ref. 120).

URIST: That is another interesting problem.

PRITCHARD: Surely, it depends on the environment. If you can put them back into the environment that they came from—

URIST: Eventually, after many cell divisions, the cell loses osteogenic potency. It may divide 19 times, but eventually it must lose the

capacity to produce bone. It may be a question of dilution of a genetic material or cytoplasmic substance.

HOLTZER: The main difficulty in this type of problem is cell transformation, or dedifferentiation, and cell selection. Whether cells in culture reversibly or irreversibly alter their metabolic behavior, or whether a cell type originally in the minority crowds out the cell type being studied, is a very confused issue. This problem can be avoided by beginning with a pure population of cartilage cells. Then by combining autoradiography and biochemical extraction procedures, one can at least pose the question of what some cells at a given moment are doing.

For example, without going into details, postmitotic cartilage cells taken out of their matrix can be induced to reenter the mitotic cycle and, in addition, to begin to synthesize a hyaluronic-acid-like molecule. Now, if after a few days they are allowed to aggregate, they cease making DNA and revert to synthesizing chondroitin sulfate. Alternatively, if the liberated chondrocytes are maintained as monodisperse cells in culture for several weeks and then allowed to aggregate, they do not go back to synthesizing chondroitin sulfate that can be detected as metachromatic matrix. In short, the chondrocyte's progeny has a fine memory for fabricating molecules required for mitotic activity, but its memory for synthesizing chondroitin sulfate, under certain conditions, is considerably more fuzzy.

PRITCHARD: It depends on the environment. If you can put them back into the environment that they came from——

HOLTZER: We did that and in the "normal" environment of the chorioallantoic membrane or the somite, they do not revert to making chondroitin sulfate. On the other hand, under conditions we have not used, they might resume their original or, for that matter, a quite novel metabolic activity. For, although the story is by no means complete, experiments on nuclear transplantations and on virus fused cells all point to a great deal of "reversible" behavior in mature cells.

URIST: Perhaps it is necessary to assume that the capacity to produce bone is lost only temporarily. Every connective tissue cell in the body may have the capacity to make bone, especially if it undergoes a series of mitotic divisions and is in a conducive environment.

PRITCHARD: That is an act of faith.

URIST: We will discuss the subject of potency again when we get to the induction systems.

Every connective tissue cell came from an original cell, so it is just a question of how far back we want to go to retrace the development from the unspecialized connective tissue cell to the osteoprogenitor cell.

PRITCHARD: Why do not liver cells make bone?



NICHOLS: Could we ask Dr. Holtzer to tell us more about his cartilage cell? Fundamentally, I agree with him; we ought to find out what cells are doing.

PRITCHARD: Dr. Holtzer says that in 12 hours they start making hyaluronic acid instead of chondroitin sulfate. Do they make collagen up to then? Could we see his figures?

HOLTZER: Many of the issues that have been discussed can only be analyzed critically with a homogeneous population of cells. Bone, at best, consists of a variety of cell types, and any measurements either of whole bone or on cells from bone, of necessity, involve measuring changes in the activity of more nonbone cells than true osteocytes or osteoblasts. By stripping away the adhering connective tissues from embryonic cartilages and treating such cartilages with trypsin to digest the matrix, a quite pure population of chondrocytes can be obtained; with care, well over 99 percent of the liberated cells are differentiated, working chondrocytes. Now, what these liberated postmitotic chondrocytes, which are making chondroitin sulfate and collagen, will do after removal from their matrix depends on how they are grown. In different microenvironments they synthesize different kinds of molecules. If, as already mentioned, liberated chondrocytes are spun down into a smaller cluster, they remain postmitotic and continue to synthesize chondroitin sulfate and collagen (ref. 121). Alternatively, if plated on top of a clot, they spread and are induced to reenter the mitotic cycle.

Figures 63, 64, and 65 show that in addition to making DNA and the other kinds of molecules, multiplying cells synthesize, dividing chondrocytes, and their progeny synthesize a polysaccharide rich in glucosamine, this polysaccharide is not sulfated and has the electrophoretic mobility of hyaluronic acid. Neither the *in vivo* chondrocytes from 10-day embryos nor the liberated chondrocytes spun down into pellets synthesize this hyaluronic-acid-like polysaccharide.

With time these cultures become more dense owing to cell multiplication. Correlated with this is a shift in the kinds of polysaccharides these cells produce. A polysaccharide appears with the mobility of hyaluronic acid but with a high galactosamine-to-glucosamine ratio (ref. 122). In addition, large amounts of chondroitin sulfate are made in the new dense cultures.

If the progeny of chondrocytes are grown for a considerable time *in vitro* and then challenged to display their capacity for synthesizing chondroitin sulfate, the results again depend on the nature of their *in vitro* microenvironment. For example, as shown in figure 66, if grown in reasonably high densities for four generations and then spun down and grown as cells in a pellet, the progeny of chondrocytes make

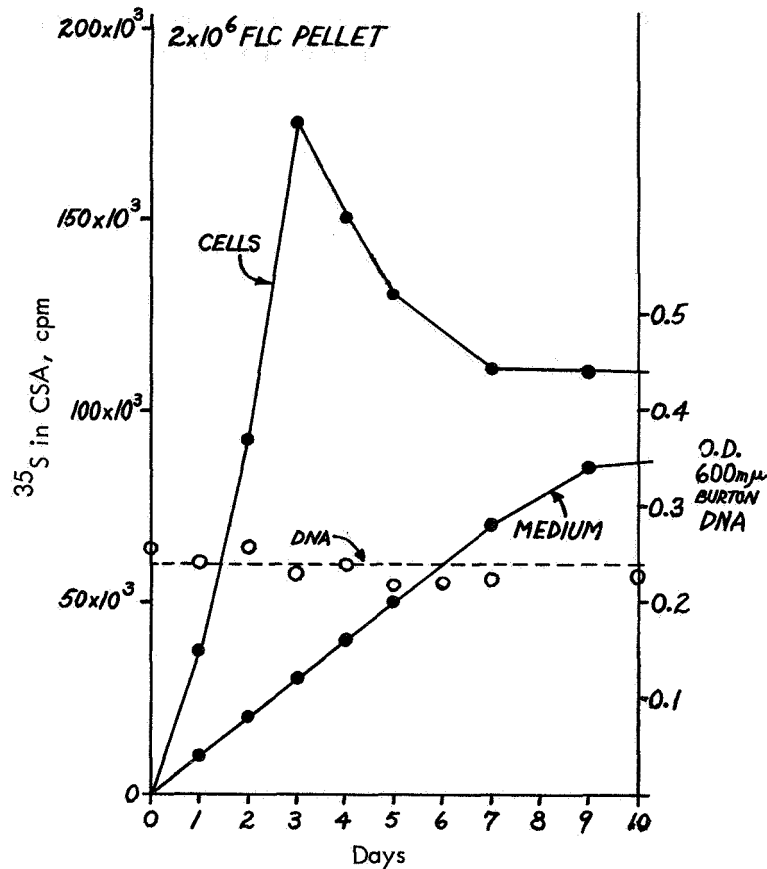


FIGURE 63. Freshly liberated chondrocytes (FLC) were spun down into a pellet and organ cultured in  $^{35}\text{S}$ . The tissues were harvested on different days and the counts in chondroitin sulfate (CSA) determined. Under these conditions there is no net synthesis of DNA and the chondrocytes continue to make chondroitin sulfate. In other experiments it has been shown that they continue to make collagen as well.

much less chondroitin sulfate per unit of DNA than freshly liberated chondrocytes grown under identical conditions.

To check whether this diminution is a result of all cells making less chondroitin sulfate per cell or whether some cells are not producing while a minority are working hard, we performed cloning experiments (ref. 123). Without going into details of culture procedures, we grew in the same dish in the presence of  $^{14}\text{C}$ -glucose, colonies of matrix producers and colonies of dedifferentiated, or transformed, chondrocytes. The matrix producers synthesized chondroitin sulfate, and the transformed cells produced a different spectrum of polysaccharides.

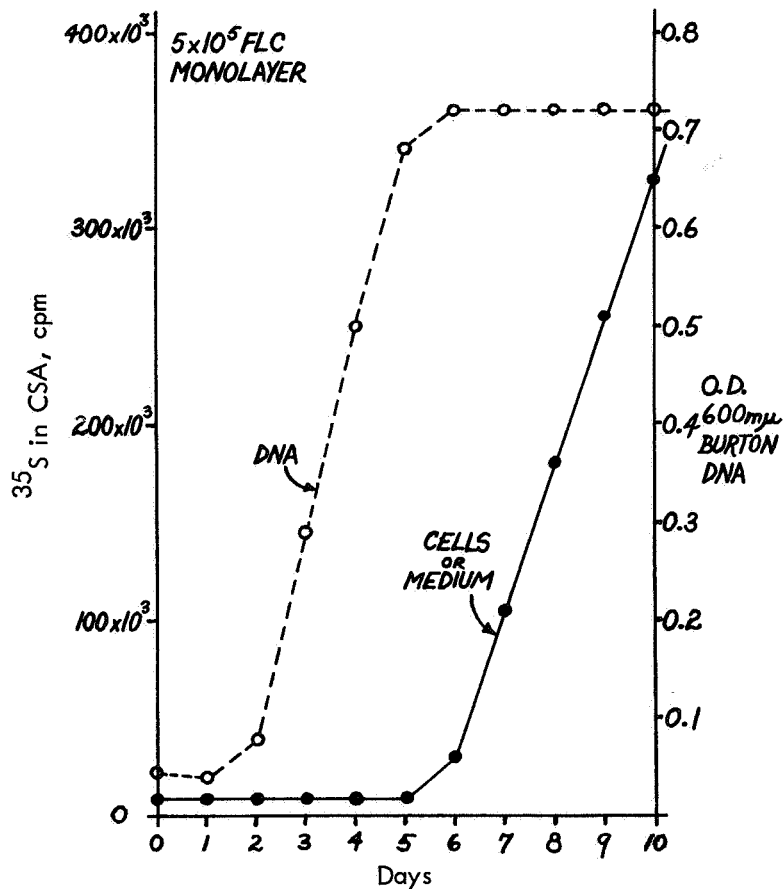


FIGURE 64. FLC cells were plated in milk bottles on top of a clot as monodispersed cells. There is an abrupt rise in DNA synthesis during the first 4 days of culture, which levels off after a certain density is reached. About this time the cells resume the synthesis of chondroitin sulfate.

From this we suspect that the chondroitin sulfate made in the fourth-generation cultures (figs. 66 and 67) might be made by a minority of the cells in the cultures; the majority are dedifferentiated, or transformed, chondrocytes. Thus far we have not been successful in shunting the dedifferentiated, or transformed, chondrocytes back into chondroitin sulfate producers.

These clonal experiments (ref. 121) led to another finding. When chondrocytes are cloned on plastic they may organize into an epithelial sheet which morphologically and functionally serves as an *in vitro* perichondrium. Cloned cells on plastic divide and, because daughter cells do not migrate, establish compact epithelial colonies. By mitosis, new cells are added to these tight little islands both in the plane of the

**ELECTROPHORESIS IN PYRIDINIUM FORMATE, pH 3.0, 500 VOLTS  
90 MINUTES, 0°C, CELLULOSE ACETATE  
0-5 DAYS FLC MONOLAYER**

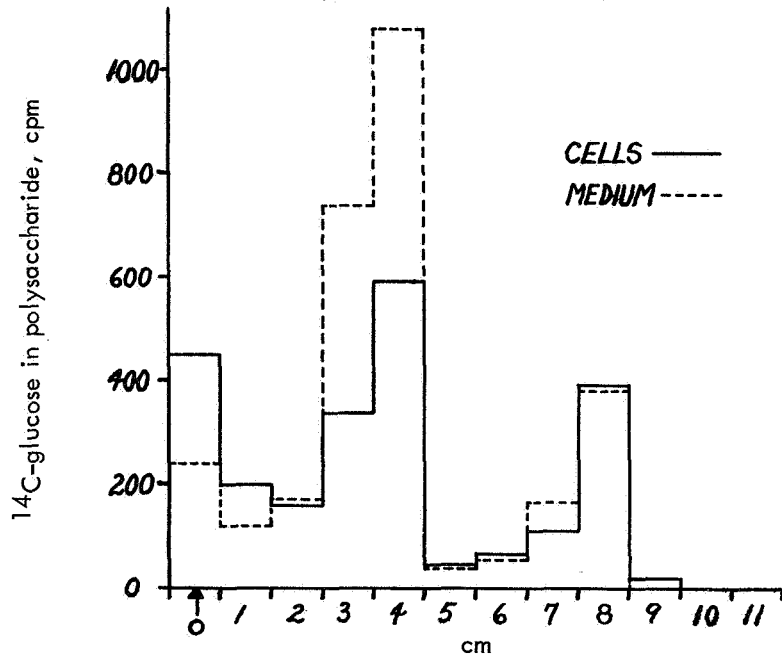


FIGURE 65. FLC cells were grown as monodispersed cells in the presence of  $^{14}\text{C}$ -glucose. After Pronase digestion the polysaccharides were separated in pyridinium formate on cellulose acetate. Observe the prominent peak where hyaluronic acid would run. On hydrolysis this peak yields a fraction with a high glucosamine-to-galactosamine ratio.

substrate and also displaced upward from the substrate. The displaced cells become rounded and synthesize chondroitin sulfate. The same cells on a fibrin clot spread, do not form epithelial colonies, and do not synthesize chondroitin sulfate in appreciable amounts. If, after growing on a clot for five generations, these cells are cloned on plastic, they fail to form epithelial colonies and fail to synthesize detectable amounts of chondroitin sulfate.

The central question to which these kinds of experiments are directed is, What kinds or species of molecules can a cell make concurrently? For example, I do not believe that a cell can make myosin and albumin simultaneously, that the cytoplasmic-nuclear conditions required for the one preclude the fabrication of the other. On the other hand, a cell synthesizing myosin does make the cytochromes, dehydrogenases, myoglobin, ribosomal proteins, glycogen, and so forth. But it is by no means clear that all kinds of molecules found in a given cell can be made

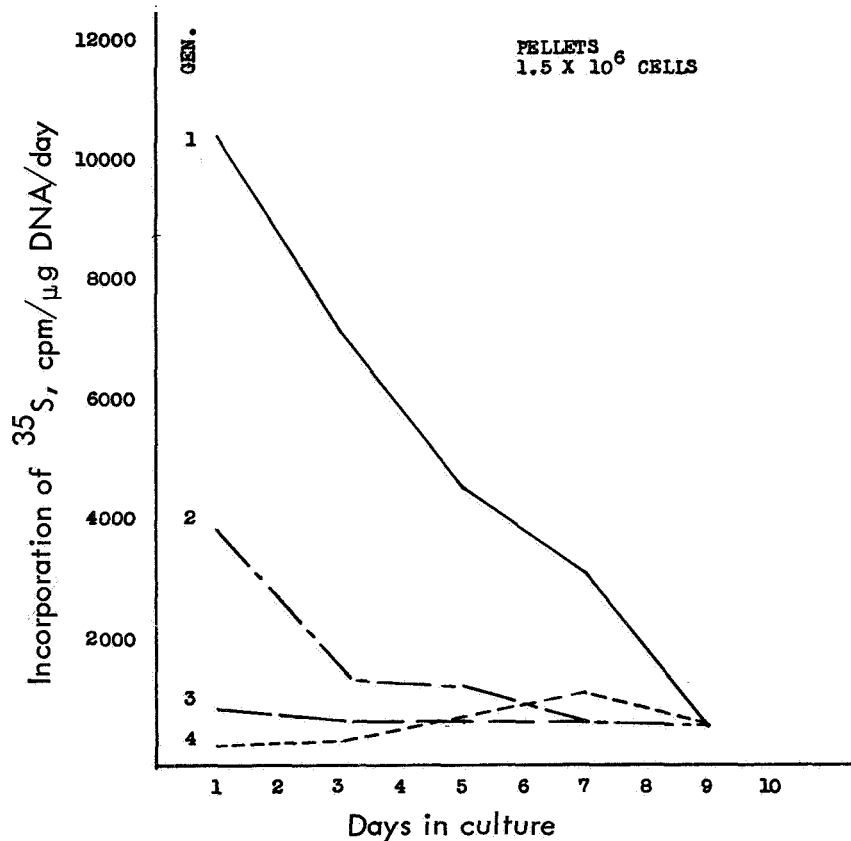


FIGURE 66. An aliquot of FLC cells was spun down into a pellet, the remainder grown as cells in monolayers. After 5 days in a monolayer situation, the cells were again divided into two groups: one grown as pellets, and the other as monolayers. After another 5 days the monolayers were again divided into two groups: pellets and monolayers. This was repeated for a third time. The values shown in this figure are for the pellets of the first, second, third, and fourth generations of cells. Clearly the amount of chondroitin sulfate made by cells in pellets varies with their previous *in vitro* history.

concurrently. For example, by definition a cell only makes DNA during S. During G-1, G-2, or M, a cell does not synthesize DNA, although clearly it is busy synthesizing other molecules. Are, in fact, all the mitochondrial enzymes, RNA's, and structural proteins made at any time in the mitotic cycle or at any time in the life history of the cell? Recently, we have shown (refs. 124 and 125) that myosin is not synthesized during S, G-2, M, or even during the first 3 to 5 hours in G-1.

Returning to pure populations of chondrocytes, we would like to know what kinds of molecules they or their progeny can make and how

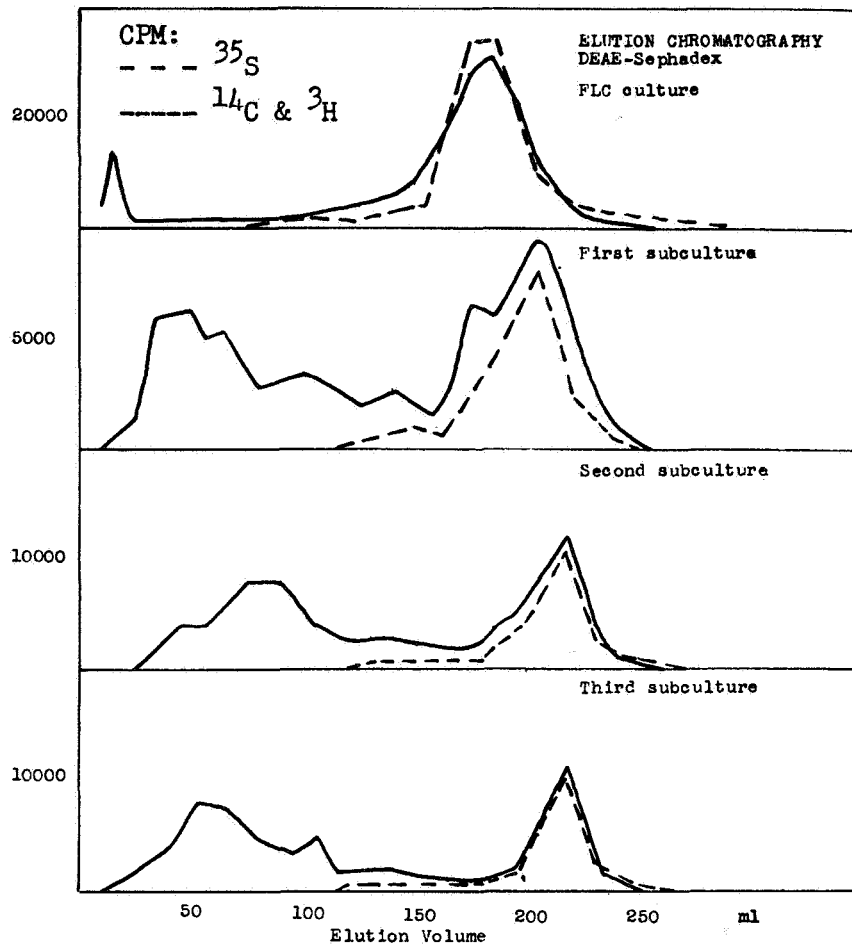


FIGURE 67. FLC cells were grown for three subcultures as monolayers and their polysaccharides analyzed by chromatography. Note that the relative amount of sulfated polysaccharide to total polysaccharide is less in the subcultured cells.

seemingly “trivial” differences in their microenvironments induce them to alter their typical products?

At this point we are not convinced that the genetic controls regulating the synthesis of, for example, amino acids or the enzymes associated with oxidative phosphorylation, are the same kind of controls that regulate the production of cell-unique molecules like chondroitin sulfate or myosin.

ROBINSON: Can you give these cells messages so that they will change—

HOLTZER: We have tried. We put them back into the animal, as you suggested, and they do not go back into the cartilage.

ROBINSON: I was thinking of the classic experiments with bacteria.

HOLTZER: No; there is no tissue system in which anybody has instructed cells to make anything. I would make that statement categorically; I said tissue cells.

NICHOLS: But you have informed these cells, because when you plated them you were placing them under a new set of conditions, if you like; you were subjecting them to a new set of stresses which was interpreted by the cells as a stimulus to do something different from what they were doing before. This is an extremely important point that we have ignored. For example, how does a bone cell know that it has been in a fracture site?

HOLTZER: That is a beautiful question.

PRITCHARD: Have you taken these cells and tried to cluster them again?

HOLTZER: Yes.

URIST: We have done a similar experiment. We have transplanted a pack of chondrocytes back into the donor, and the cells do not resume chondrogenesis.

MCLEAN: I want to throw a monkey wrench into this machinery we have been talking about. It is not mine but something that Frost (ref. 118) has published; he calls it a concept. He starts with a line of stem cells, and then some of these stem cells divide. He has introduced the idea that every time a stem cell divides, it produces an undifferentiated reserve cell and simultaneously produces one differentiated cell. For instance, a stem cell could give rise to an osteoblast and another stem cell.

PRITCHARD: I would like Dr. Young to comment on this because he has been concerned with these relationships as much as anyone else; and Dr. Owen, too.

HOWELL: Would Dr. Young also comment on whether cell death is part of this picture?

PRITCHARD: That these eventually finish their life cycle and die.

YOUNG: I think most of us who have been studying normal bone growth have seen practically no cell death. I would rather address my comments to the important point, which grew out of Dr. Holtzer's presentation and was seconded by some others, that the critical information for these various activities is already in the cell, coded in its DNA. One need not provide the cell with information-bearing macromolecules, as in the transformation experiments, to call forth from it potentialities which it might not have shown in the living organism.

I prefer to think, although we are not down to control mechanisms, that the effect of the microenvironment is among the most important

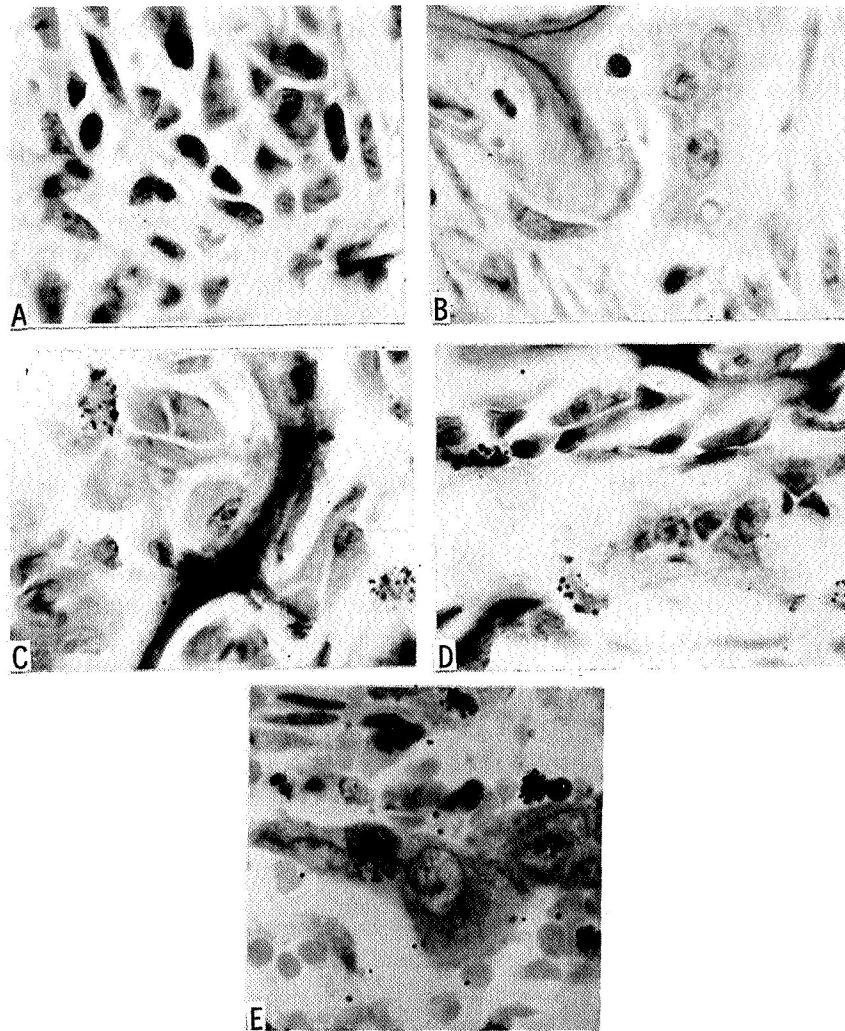


FIGURE 68. Photomicrographs of sections of tibial metaphysis of 1-week-old rats. PAS-hematoxylin stain. 920 $\times$ .

(a) Osteoblasts and osteoprogenitors are seen in this field. Note the two osteoprogenitors in prophase near the center.

(b) An osteoclast and several osteoprogenitors are visible.

(c) One hour after injection of <sup>3</sup>H-thymidine. Several osteoprogenitors are labeled, indicating DNA synthesis prior to mitosis. Osteoblasts are unreactive. Autoradiograph.

(d) One hour after injection of <sup>3</sup>H-thymidine. Labeled nuclei are seen in osteoprogenitors, but not in the osteoclast (center). Autoradiograph.

(e) Sixteen hours after injection of <sup>3</sup>H-thymidine. Labeled nuclei are now found in osteoclasts, indicating that these cells are derived through specialization of osteoprogenitors. Autoradiograph.



factors in the control mechanism. While on this point I would like to show some figures.

One of the requirements for working with tritiated thymidine is to be able to classify the cells. Figure 68(a) shows the cells, the osteoblasts, that one sees around the bone. However, there are also pale-staining, rather fusiform cells, which I call osteoprogenitors.

Figure 68(b) is of another region and shows an osteoclast; again there are several of the pale-staining cells, the osteoprogenitors.

Work with tritiated thymidine has been repeated in many laboratories. It shows that osteoblasts and osteoclasts are incapable of reproducing themselves. They do not take up thymidine, they do not synthesize DNA, and they do not divide (refs. 126 and 127).

However, shortly after the injection of tritiated thymidine, we find that the cells which are synthesizing DNA and will divide are the pale-staining cells, the osteoprogenitors, as shown in figure 68(c). The specialized osteoblasts rarely take up thymidine.

Figure 68(d) is of another field and shows an osteoclast; again, synthesis of DNA and preparation for cell division are occurring in the pale-staining osteoprogenitors. Shortly after the completion of DNA synthesis, these cells divide. We can keep track of them because they are radioactive.

Figure 69 is a scheme of the cell cycle. We have made radioactive those cells that were in the DNA synthetic period (S) at the time of injection; and if we now, in a series of intervals thereafter, continually sample this histologically recognizable mitotic compartment (M), we will see the passage of cells from DNA synthesis into mitosis. There is a peak that represents this division, which has occurred within a few hours after DNA synthesis; some of these cells will still continue to divide (refs. 109 and 128).

This can be determined in any number of ways. One simple way is to watch them come through a second time. They are becoming more uniformly distributed temporarily throughout the cycle.

PRITCHARD: This percentage of labeled cell refers to what total population?

YOUNG: We searched the region of bone for mitotic cells and recorded the percentage of those that are radioactive.

PRITCHARD: Relative to every cell in the neighborhood?

YOUNG: No; just looking at the dividing cells. It is a percentage of all the mitoses that were radioactive, and at this interval every dividing cell is radioactive.

The point is that the labeled progenitor cells do divide. That is why they were synthesizing DNA in the first place; they were getting ready to divide. Some of them continue to divide. Others, however, change their specialization, reorganizing their metabolic machinery

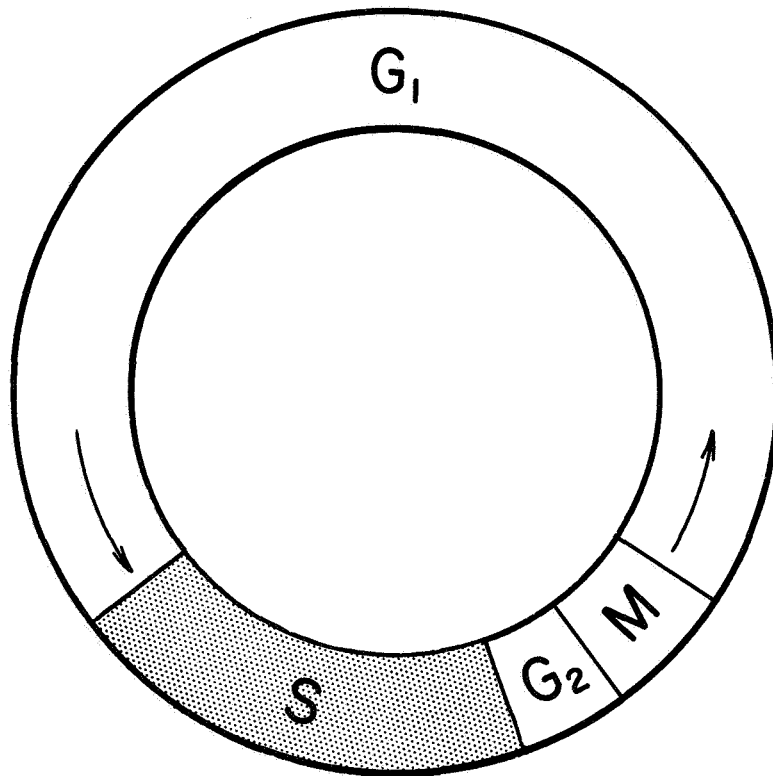


FIGURE 69. A schematic representation of the cell cycle in a dividing population. Interphase is subdivided into  $G_1$ , DNA synthesis (S), and  $G_2$  periods. Cells engaged in DNA synthesis in the presence of  $^3\text{H}$ -thymidine become radioactive. These labeled cells may then be followed through the histologically recognizable mitosis (M) phase during subsequent hours.

to become the specialized cells that we discussed previously; so that within a few hours, we can begin to detect radioactive nuclei in osteoblasts. Although the thymidine is available for only about an hour, none of these cells is initially labeled. Only after the osteoprogenitors have had time to specialize do we begin to pick up increasing numbers of labeled nuclei in osteoblasts.

Within a few hours one begins to find labeled nuclei also in osteoclasts (fig. 68(e)); this demonstrates that the osteoprogenitors can continue to divide or, depending upon the microenvironment in which they find themselves, may specialize as either osteoblasts or osteoclasts.

Ultimately, some of the osteoblasts become trapped in bone, in which case we then see labeled nuclei in the same cell; but now we call it an osteocyte.

I made the statement that osteoprogenitors are equally capable of specializing as osteoblasts or osteoclasts, and I would like to try to document that in several ways. (The quantitative data are given in detail by Young (ref. 128).) In young growing rat bone, the percentage of progenitor cells initially labeled is on the order of 25, 15, and 5 percent in metaphysis, endosteum, and periosteum, respectively.

If we chart the gradual appearance of labeled nuclei in osteoclasts, we find that in the metaphysis, the proportion peaks and will not exceed about 25 percent, in the endosteum it does not exceed 15 percent, and in the periosteum, 5 percent. The same analysis can be made for osteoblasts. The numbers are even more convincing. In the metaphysis the proportion peaks at about 25 percent, in the endosteum at about 15 percent, and in the periosteum, at about 5 percent.

In an effort to demonstrate that these cell specializations can be affected by the microenvironment, which is not a very daring statement, we can alter the environment one way or the other with different experimentally induced changes. In this regard, I have investigated the influence of parathyroid extract (fig. 70(a)). The remarkable increase in bone resorption in the treated animal is accompanied by rapid changes in cell specialization.

At the beginning of the experiment in the young rat, we see the normal picture (fig. 70(b)) with large numbers of osteoblasts. Within 4 or 5 hours after injection, we begin to see changes in the morphology of the cells. At 12 hours (fig. 70(c)), some cells can still be classified as osteoblasts, but some are beginning to assume the morphology of the progenitor cells.

A few hours later (fig. 70(d)) there are large numbers of osteoclasts forming by specialization from the precursor. There are essentially no osteoblasts under these high doses, but there are a large number of osteoprogenitors.

At the height of the effect of the hormone, which in these young rats is about 22 hours after injection (fig. 70(e)), we find large numbers of osteoprogenitors and no osteoblasts. When we provide these osteoprogenitors with tritiated glycine, we may recall how greatly glycine was concentrated on osteoblasts (see fig. 60). In contrast, these cells not only look like osteoprogenitors, they are behaving like osteoprogenitors, as shown by their diminished utilization of the labeled glycine. In fact, they are osteoprogenitors.

PRITCHARD: May I ask what are the cells along the spicule if they are not osteoblasts?

YOUNG: I think by the absence of any cytoplasmic basophilia whatsoever and by their very low utilization of glycine—

PRITCHARD: They are simply not working. They are inactive osteoblasts. Why not?

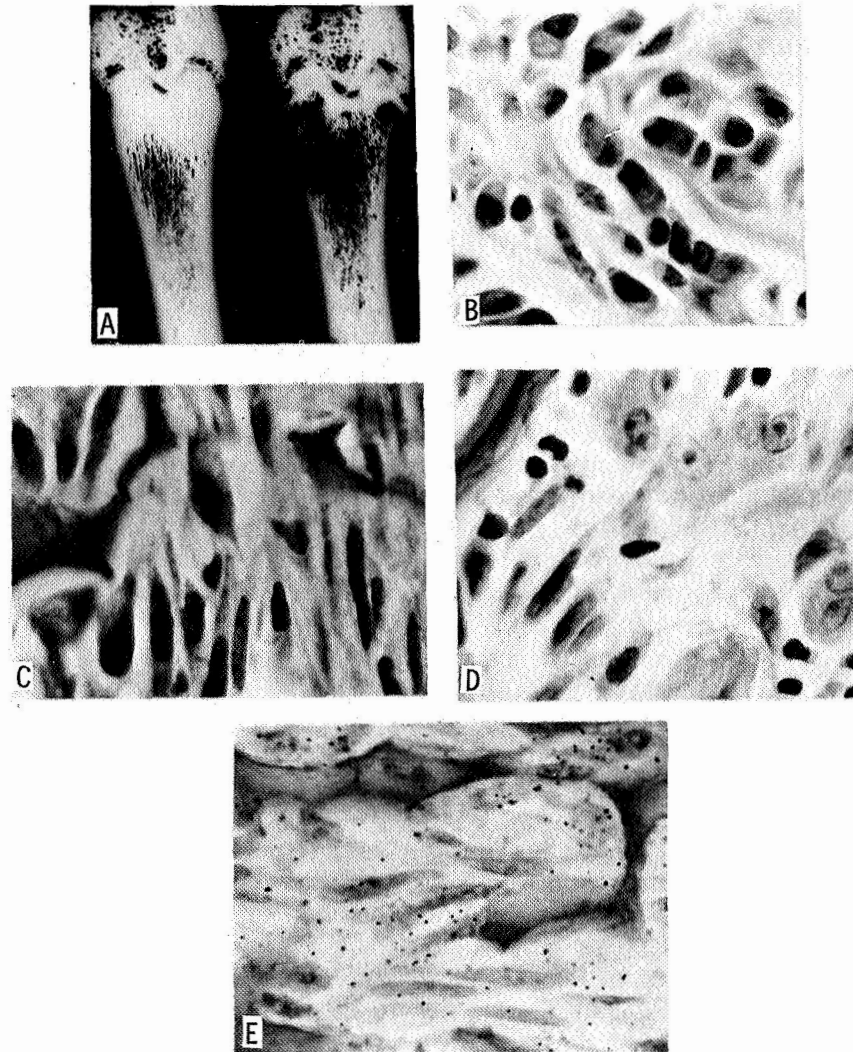


Figure 70. Effect of parathyroid extract (PTE) on rat tibias.

(a) Roentgenograph of tibias of 4-week-old rats. Control (left) and sacrificed 48 hours after large dose of PTE (right). Note the marked resorption in metaphyseal region.

Photomicrographs of sections of tibial metaphysis of 1-week old rats. PAS-hematoxylin stain. 870x.

(b) Prior to onset of treatment; large numbers of osteoblasts are present.

(c) Twelve hours after PTE. Osteoblasts in process of returning to osteoprogenitor state under changed microenvironment.

(d) Eighteen hours after PTE. Most of bone cells are now specialized as either osteoprogenitors or osteoclasts.

(e) Twenty-two hours after PTE and 30 minutes after injection of  $^3\text{H}$ -glycine. Osteoprogenitor cells, formed through respecialization of osteoblasts under the influence of PTE, fail to concentrate labeled glycine as they did in the osteoblastic state. Autoradiograph.

YOUNG: I prefer to call them osteoprogenitors. If, in the same experiment, we provide these cells with tritiated thymidine, all of these cells appear to be in the osteoprogenitor state.

Table IX is an effort to put this effect in quantitative terms. The grain-count ratio represents, in a glycine-treated animal, the ratio of silver grains over osteoblasts compared with those over osteoclasts. As I tried to demonstrate earlier, there is an enormous preponderance of glycine utilization in osteoblasts as compared with osteoclasts.

TABLE IX  
PERCENTAGE OF  $^3\text{H}$ -THYMIDINE-LABELED NUCLEI IN OSTEOLASTS AND OSTEOLASTS  
IN RIBS AND TIBIAS OF RAT <sup>a</sup>

Experimental data	Osteoblasts		Osteoclasts	
	Rib	Tibia	Rib	Tibia
Normal animals.....	23	16	12	10
PTE, given 1 to 7 hours after $^3\text{H}$ -thymidine injection.....	2	1	12	11
PTE, given 12 to 24 hours before $^3\text{H}$ -thymidine injection.....	26	15	1	1

<sup>a</sup> Averaged percentages, taken from data reported by Young (refs. 109 and 111).

Parathyroid extract is given at the beginning of the experiment. The rats are sacrificed at different intervals thereafter. As the osteoblast undergoes morphologic changes back to the osteoprogenitor state, there is a rapid decrease in this physiologic measurement of the protein synthetic activity of these cells. At about 22 hours after injection the cells look like osteoprogenitors, behave like osteoprogenitors with respect to thymidine, and behave like osteoprogenitors with respect to glycine. One is led to conclude that they are indeed osteoprogenitors.

If we allow the animals to recover, within about 2 days after the height of the effect of the parathyroid extract, the cells will have returned to their normal behavior. The osteoblasts are repairing the damage; and throughout these rapid changes in cell specialization, there is no observable increase in cell death. I think this table is an important one. The experiments pose the following two questions. Are the progenitor cells capable of specializing as osteoblasts or osteoclasts? Can we influence this specialization by changing the microenvironment of the cells? I think the answer to both of these questions is "Yes."

If the normal animal, the rat in this case, is injected with tritiated thymidine, the initially labeled cells are the osteoprogenitors. If we allow the animal to survive to a day or so after injection, we find that some of the osteoprogenitors have specialized as osteoclasts and others have specialized as osteoblasts, depending upon their location in the bone and the microenvironmental stimuli which were acting upon them.

If, instead, we follow the thymidine treatment by a large dose of parathyroid extract, we find that, a day or so later, large numbers of the initially labeled osteoprogenitors have specialized as osteoclasts. Very few osteoblasts have been formed under these conditions of high levels of parathyroid extract in the cellular environment.

In the third experiment, we first pretreat the animal with parathyroid extract to induce the osteoblasts to revert to the osteoprogenitor state. If, at that time (when the bone is filled with osteoclasts and progenitors) we provide the animal with thymidine to label the osteoprogenitors, and then allow it to recover, we find that during the recovery period the cells preferentially specialize as osteoblasts.

So, I think that these are different functional states of the same cell, and that the specialization of the progenitor cell in either one or the other direction (as well as in various additional directions which I have not had the opportunity to discuss), is determined by the immediate microenvironmental circumstances in which the cell finds itself.

PRITCHARD: There are two important concepts here. One is the reversibility of this chain of cells, and the other is the effect of parathyroid hormone on the cell population. I do not know whether Dr. Owen wants to say anything about the first question of reversibility—

OWEN: No. I think Dr. Young's experiments are very elegant.

URIST: Dr. Pritchard has made two important points this morning; one, that there is a classification problem; the other is the target cell for hormones that affect bone. Is the progenitor cell the target cell for parathyroid hormone? What is the target cell for thyrocalcitonin?

PRITCHARD: I think Dr. Talmage has some ideas on this.

TALMAGE: I have a few figures.

URIST: First, I would like to ask Dr. McLean to show his figure; there is just one.

MCLEAN: I would like to ask Dr. Young, are these experiments all on rats?

YOUNG: Yes.

MCLEAN: I would like to point out that the reaction of the rat to parathyroid hormone is different from that of any other mammal that I know anything about. It is possible with relatively low doses of parathyroid extract to produce this state of hyperostosis, to make everything differentiate into osteoblasts without ever going through

the stage of increased resorption and increased osteoclastic differentiation.

YOUNG: With repeated low doses of beef parathyroid extract, experimental animals may produce antibodies against the foreign protein, while the chronic doses may also inhibit the endogenous parathyroid secretion (ref. 129).

MCLEAN: The only thing I am saying is that your statistics are for the rat, and that represents a different cycle from what one will find in any other animal.

YOUNG: The absolute numbers of how many progenitor cells are labeled is unimportant; it does not matter. Parathyroid extract is used here only as a tool to demonstrate the interconversions of the cells, which can be demonstrated in many ways. I was not studying the effect of parathyroid extract from the standpoint that Dr. Talmage might be.

MCLEAN: My point is that this selective differentiation into osteoclasts or osteoblasts according to the time that you give the parathyroid extract is something special for the rat.

YOUNG: I do not believe that is true, Dr. McLean. The formation of increased numbers of osteoprogenitors and osteoclasts in response to a single, large dose of parathyroid extract is very striking in the young rat, but is by no means unique in this animal. For example, it has also been observed in young guinea pigs (ref. 130), mice (ref. 131), dogs (refs. 132 and 133), and pigeons (ref. 134).

The preferential formation of osteoblasts during the recovery phase after a single, large dose of parathyroid extract (refs. 111 and 135) represents a return toward the normal condition.

On the other hand, if small doses of the extract are repeatedly administered to the rat, it is indeed possible to obtain a preferential and exaggerated formation of osteoblasts without previous increase in osteoclast formation (ref. 136) or following a transient increase in the numbers of osteoclasts (refs. 129, 137 and 138). A similar phenomenon has been reported in the mouse (ref. 139), indicating that the rat is not unique in this respect either.

ROWLAND: Dr. McLean, do you have experimental evidence for other species to contradict these experiments?

MCLEAN: Yes. Years ago, when we were working very actively on parathyroid extract, we tried to reproduce these phenomena in other animals. We ran through the whole gamut of experimental animals. We never saw this hyperostosis, as we were calling it then, except in the rat.

YOUNG: I believe the significant thing about these experiments is the demonstration that these cell interconversions take place. If we set aside parathyroid extract and looked at the work that came out

of your laboratory, Dr. McLean, some of the early work on cell interconversions in birds during the egg-laying cycle, we would find these studies demonstrated precisely the same thing, the interconversion of the cells of the bone series. The osteoprogenitors were at that time called, I think, reticular cells.

MCLEAN: But that was not parathyroid extract.

YOUNG: No; it was not, but it demonstrated the same basic phenomenon of cell respecialization which to me is the most important point.

MCLEAN: I agree with that. During the experiments we did many years ago (ref. 140), we ran into a phenomenon, also peculiar to the rat, of cell death under the influence of large doses of parathyroid extract (see fig. 111).

We observed osteocytes with pyknotic, or disintegrated, nuclei in rats 12 hours after intraperitoneal injection of 1000 units of PTE, a highly toxic dose; we were quite excited about this at the time. We thought it was the mode of action of the parathyroid hormone (ref. 133). We never were able to duplicate this, however, in any other animal (ref. 135).

In animals in the same series, this effect was followed by the stage of hyperostosis, new bone building, and differentiation of large numbers of osteoprogenitor cells into osteoblasts. The first stage is osteoclastic with the peculiar phenomenon of cell death.

URIST: Thank you. Now, Dr. Talmage.

FREMONT-SMITH: You must explain why my tissue culture idea is a red herring as well as a grunion.

TALMAGE: I would like to leave it to Dr. Raisz to explain the reasons tissue cultures using bone are subject to misinterpretation, since he is an expert in this field. The primary problem is that as yet no one has been successful in getting a pure culture of one type of bone cells. Using cartilage, the situation may be different; so discussions based on tissue culture of cartilage cells may not be apropos to bone problems.

But I would like to change the subject now, and discuss the influence of parathyroid hormone on osteoclasts. Let me say at the beginning that all our work was done using rats. The fact that experts have questioned the use of rats because the bone of this species is different from that found in either man or dog actually bolsters my argument. My primary thesis is that the effect of parathyroid hormone is not on osteoclasts, but on the formation of osteoclasts. Since the rat is the only species in which one can really show this action of the hormone on osteoclasts, this bolsters, even further, my argument that calcium homeostasis is not a function of the osteoclast. However, the data which I would now like to present are a demonstration of the site of



action of the parathyroid hormone in stimulating the production of osteoclasts in the rat.

URIST: What is the target cell for parathyroid hormone?

TALMAGE: It is the mesenchymal cell. In the matter of terminology, we feel that because the term "mesenchymal cell" is an older term it should have preference; we should use the older terms unless we have good reasons for changing them. Otherwise, I do not object to Dr. Young's "progenitor" cells.

The first point is to demonstrate, what all of us must know, that in the rat there is a marked effect of parathyroid hormone on osteoclast production.

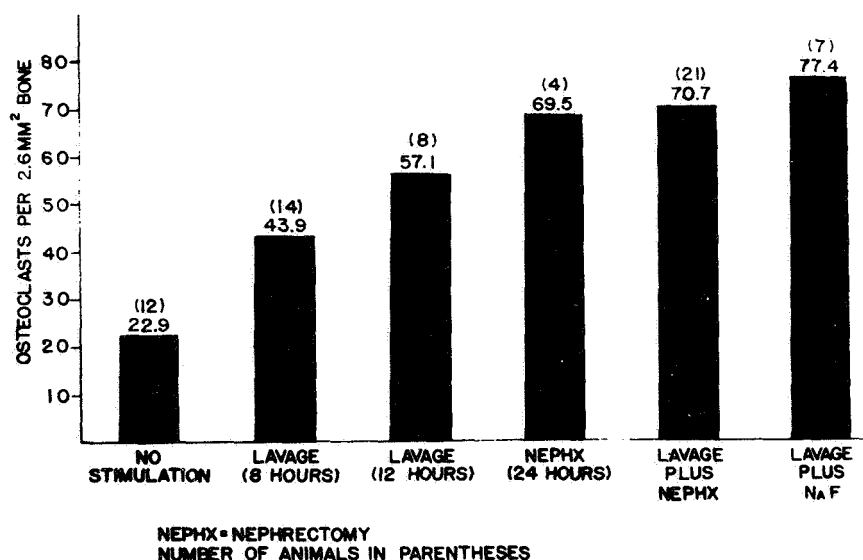


FIGURE 71. Methods of increasing osteoclast numbers in the metaphysis of the femur. [From Talmage et al. (ref. 141); reprinted by permission of the publisher.]

Figure 71 is from an earlier publication (ref. 141). It merely recalls for you the fact that one can quantitate the increase in osteoclasts in the metaphysis of the femur of the rat as a function of parathyroid activity. The normal osteoclast count is given at the left. After stimulation by peritoneal lavage with a calcium-free rinse, which is one of the best ways to increase endogenous production of parathyroid hormone, it is easy enough to show that there is a direct relation between the number of osteoclasts and endogenous parathyroid secretion.

Another method for stimulating secretion is to nephrectomize the animal. This, because of the subsequent rise in phosphate, causes a

decrease in calcium and therefore a stimulation of the animal's parathyroids. This increases the osteoclast count.

The method by which we produced the largest increase in number of osteoclasts, as the result of increasing the animal's own endogenous hormone production, was to use a calcium-free lavage containing fluoride. This fluoride produced a decrease in the solubility of the bone mineral. This resulted in an increased stimulus to the parathyroids. With this background, let us look at the source of these new osteoclasts.

TABLE X  
EFFECT OF PERITONEAL LAVAGE ON PERCENT NUCLEI LABELED 48 HOURS AFTER  
<sup>3</sup>H-THYMIDINE INJECTION

[Adapted from Talmage et al. (ref. 141)]

Group	Osteoclasts	Osteoblasts
Normal animals.....	10.6 ± 0.48	20.2 ± 1.01
Normal, lavaged.....	1.8 ± 0.42	18.0 ± 0.68
PTX animals <sup>a</sup> .....	9.8 ± 0.83	20.8 ± 0.66
PTX, lavaged <sup>a</sup> .....	11.3 ± 0.44	19.3 ± 0.68
Average of all groups:		
Osteoblast: 18.8 ± 0.91		
Mesenchymal cells: 6.3 ± 0.48		

<sup>a</sup> PTX = parathyroidectomy.

The data in table X are based on Dr. Young's work. Dr. Young just demonstrated that 48 hours after tritiated thymidine injection, the labeling of mesenchymal cells goes from a high of about 25 to 30 percent back down to 6 percent. Table X indicates that our data substantiate this. Also, by that time, the percent of labeled osteoblasts is up to 18 percent, and the osteoclasts up to 10 percent. These figures agree with those given by Young.

Now, if 48 hours after <sup>3</sup>H-thymidine injection, we produced a marked increase in the number of osteoclasts by an 8-hour peritoneal lavage, we should be able to determine if the osteoclasts were derived from osteoblasts. If so, the percentage of nuclei labeled would have to end up somewhere between the starting percentage for the two types of cells. If they were to come directly from mesenchymal cells alone, one would expect labeling to be between 10 and 6 percent. But you will see that the number of osteoclasts that were labeled after this sudden burst of production of osteoclasts dropped below both that for the original osteoblasts and for the mesenchymal cells (ref. 141).

This threw us into a quandary, as we had been hoping to show that these new osteoclasts came from mesenchymal cells. However, if one assumed that most of the mesenchyme cells that produced new osteoclasts had to first go through mitosis, this would dilute the label sufficiently so that the radioactivity of thymidine would not be detectable in the osteoclasts.

While we had eliminated the osteoblast, we did not feel we had eliminated mesenchyme cells, so we shifted, then, to mesenchyme cells and a study of the uptake of tritiated cytidine.

TABLE XI  
EFFECT OF PARATHYROID STIMULATION ON PERCENT NUCLEI LABELED 30 MINUTES  
AFTER <sup>3</sup>H-CYTIDINE INJECTION

[Adapted from Talmage et al. (ref. 141)]

Group	Number	Mesenchyme cells
A. Nonlavaged: Intact.....	7	10.2 ± 0.69
B. Lavaged:		
16 hours, intact.....	7	18.2 ± 0.70
8 hours, intact.....	8	20.2 ± 0.69
16 hours, PTX <sup>a</sup> .....	7	11.5 ± 0.87
All groups.....	16	
Osteoblasts: 9.6 ± 0.59		
Osteoclasts: 4.1 ± 0.49		
Osteocytes: 1.2 ± 0.33		

<sup>a</sup> PTX = parathyroidectomized after the eighth hour.

Table XI demonstrates the labeling of mesenchyme cells with tritiated cytidine one-half hour after injection (ref. 141). We used the ½-hour period because at that time the cytidine is still in the nucleus. Examination of these data demonstrates that the stimulus for increasing endogenous parathyroid hormone secretion produced by the peritoneal lavage technique caused a doubling of the percentage of mesenchyme cells labeled. This could be inhibited by parathyroidectomy. At no time could we see any effect on the labeling of other cells; that is, the osteoblasts, the osteoclasts, or the osteocytes. Therefore, we assume that parathyroid hormone must have been affecting the RNA turnover in mesenchyme cells.

PRITCHARD: You did not have any difficulty in deciding which was a mesenchyme cell and which was not?

TALMAGE: I am sure we have the same difficulty that everybody else has. In our study we call mesenchyme cells the large group of

cells that are not lying against the bone, are mononucleated, and somewhat spindle shaped.

PRITCHARD: If it is not an osteoblast or an osteoclast, it is a mesenchyme cell.

TALMAGE: Since we are amateur histologists, if it is not an osteoclast or an osteoblast, and if it is in an area away from the bone, we usually call it a mesenchyme cell. This is a very broad term which includes all stages in the development of the cell.

PRITCHARD: You would not have counted those in the endothelium?

TALMAGE: No.

PRITCHARD: You would not have counted any hemocytoblasts you picked up?

TALMAGE: We tried not to. Therefore, we feel that we have demonstrated that what we call mesenchyme cells are the cells affected by parathyroid activity.

TABLE XII

EFFECT OF ACTINOMYCIN D ON OSTEOCLAST COUNT IN FEMORAL METAPHYSIS OF PERITONEAL LAVAGED RATS

[Adapted from Talmage et al. (ref. 142)]

Group	Count
Controls, nonlavaged (12).....	23.7 ± 1.2
Controls, lavaged 8 hours (10).....	53.8 ± 1.7
PTX, lavaged 8 hours (6).....	32.6 ± 1.6
AMD, injected 8 hours before lavage (6).....	37.1 ± 4.2
AMD, added to lavage rinse (6).....	56.6 ± 4.0

Numbers in parentheses indicate number of animals.

The data in table XII back up this assumption (ref. 142). In our lavage system, when we added actinomycin D to the lavage fluid, it caused a fall in the rate of calcium removal by the fourth or fifth hour. The important point here is that adding the drug to the fluid did not prevent the normal increase in osteoclasts which occurs with lavage. Normal increase is between 50 to 100 percent in 8 hours. When, however, the drug is given 8 hours prior to the start of the lavage, it prevents this increase in the number of osteoclasts formed.

This suggested to us that whatever parathyroid did, it did immediately, before actinomycin D could knock out RNA production. Despite the presence of the drug, the simultaneous stimulation by endogenous hormone was still able to produce osteoclasts. I think this is a very important consideration, as it indicates the rapidity of the action of the hormone.

TABLE XIII

EFFECT OF  $^{239}\text{Pu}$  ON OSTEOCLAST COUNT AND  $^3\text{H}$ -THYMIDINE-LABELED MESENCHYME CELLS IN RAT FEMORAL METAPHYSIS

[From Doty et al. (ref. 143)]

Group	Number of animals	Osteoclasts per field	Number of animals	Mesenchyme cells $^3\text{H}$ -thymidine-labeled, percent
Control animals.....	11	$33.0 \pm 1.5$	3	$19.2 \pm 0.6$
$^{239}\text{Pu}$ , 24 hours.....	7	$35.4 \pm 2.3$	3	<sup>a</sup> $12.0 \pm 0.5$
Control, lavaged.....	14	$54.1 \pm 2.6$	.....	.....
$^{239}\text{Pu}$ , lavaged.....	12	<sup>b</sup> $41.9 \pm 1.6$	.....	.....

<sup>a</sup> Significantly lower than noninjected control animals ( $p < 0.001$ ).<sup>b</sup> Significantly lower than noninjected lavaged animals ( $p < 0.001$ ).

Table XIII demonstrates the effects of plutonium (ref. 143). We had discovered that if plutonium is injected into an animal, the first effect produced in 24 hours——

BAUER: How much?

TALMAGE: The dose was ample, 1 mg/100 g animal weight. I am not trying to stress long-range effects of plutonium. An immediate effect of plutonium is that by 24 hours the bone was unable to respond to the stimulus of endogenous parathyroid secretion, at least in that no osteoclasts were found following peritoneal lavage. This is illustrated in table XIII. Also summarized are data on the inhibition by plutonium, under these conditions, of the uptake of  $^3\text{H}$ -thymidine by mesenchyme cells. These data would again indicate that the effect of parathyroid hormone is on mesenchyme cells.

I would like to discuss figure 46 in more detail and with a different emphasis. It represents some of our recent work and is preliminary data.

In these experiments the rats were subjected to peritoneal lavage for up to 8 hours. Following lavage, the bones were dissected out as fast as possible. It was only 8 minutes from the time the animal was killed until the bone was placed into the incubation flask. Tritiated cytidine and thymidine were added to the serum and incubated with the bones for 1 hour. RNA and DNA were extracted and the specific activities were determined.

I would like to emphasize the point that the parathyroid stimulation needed to last only 20 minutes to produce a very marked increase in the uptake of  $^3\text{H}$ -cytidine by RNA. This increase was seen only in

the metaphysis during the early hours, but later during the lavage both the metaphysis and the diaphysis were affected.

The fact that the early stimulation is in the metaphysis suggests that we are seeing effects primarily in the large population of mesenchyme cells which are located in this area of bone.

I would like to use figure 46 to demonstrate one other point which we have not yet proved statistically but which may become important. In this figure, the  $^3\text{H}$ -cytidine uptake by the two types of bone in control rats is shown as the baseline in each graph. The value for the nonlabeled animal, parathyroidectomized for 18 hours, is given at the zero hour on the lavage time schedule. In the metaphysis, parathyroidectomy alone increased cytidine incorporation into RNA. There was no further effect due to lavage. This is the first time that we have been able, with our technique, to demonstrate a possible effect of endogenous parathyroid hormone on what I assume must be osteoblast function. It appears that parathyroidectomy released a suppressor of RNA synthesis. Following stimulation by peritoneal lavage, the stimulation for RNA production in the mesenchyme-cell population reversed this suppression and  $^3\text{H}$ -cytidine uptake increased. This, of course, is speculation.

The last point that I would like to emphasize is that there is a marked difference between the effects in the metaphysis and those in the diaphysis. This was mentioned briefly in Session I. If we are correct in assuming, as we have indicated before, that the calcium homeostatic function is centered primarily in the areas of the diaphysis, we would interpret the data in this figure to indicate that the effects on RNA production here is not a mechanism by which certain bone cells are controlling calcium homeostasis, but rather a parathyroid stimulation is causing the cell to change its orientation, the forming of new osteoclasts.

Figure 47 illustrates  $^3\text{H}$ -thymidine uptake into DNA; it is merely to demonstrate that DNA is affected by the same system. Thymidine uptake is also affected, but there is a time delay. It suggests that the parathyroid stimulus increases the mitotic rate in the mesenchyme population, causing reorientation of a certain percentage of these cells into osteoclasts.

To summarize, we believe that the stimulus of parathyroid hormone is not on the osteoclast itself, but on the mesenchyme cell population to produce more osteoclasts and that these latter cells are probably not concerned with the control of calcium homeostasis.

URIST: Thank you very much. Dr. Pritchard, I believe we have reached the point where it is clear—at least it is to me—that once a cell is differentiated as an osteoprogenitor cell, it can respond to hormones in various ways. Now can we discuss the subject of what

induces cells to differentiate into an osteoprogenitor cell; i.e., to get on Young's wheel?

PRITCHARD: I do not think we should take Young's wheel for granted. We have a population in which some cells are dividing, some are differentiating, and others are differentiated and actively engaging in matrix formation or destruction.

There is still doubt as to the reversibility of this system. I would like to see some concrete evidence that osteoclasts actually break up into progenitor cells.

There are many factors that can affect this cell system. I think Dr. Peck has information about a vitamin effect on the system and also Dr. Budy, about the effect of estrogen on bone.

RAISZ: Dr. Peck and I have been discussing the issue of tissue culture versus studies on tissue *in vivo*. And since the question has been raised and not answered, I think in fairness to the various groups around the table who are using different systems *in vivo* and *in vitro*, we ought to attempt to clarify what these different approaches can achieve. In a conference on topics as complex as this, we could become further confused by going back and forth between *in vivo* and *in vitro* data.

We are going to hear about what I regard as elegant *in vitro* data from Dr. Peck. This work proves something important about the way living cells work, but it does not prove anything about the way the precursors of these cells function *in vivo*. Dr. Fremont-Smith asked us to look at tissue culture to find answers to the questions that Dr. Young is raising. I would say that this was a wrong approach, because Dr. Young was asking an entirely descriptive question about the way in which a living system showed modulation of cells in response to external agents. I do not see how any *in vitro* system can tell you what the sequence of this cellular modulation would be. On the other hand, we can do much better experiments on the biochemistry of cell transformation *in vitro*.

FREMONT-SMITH: The point I wished to make is that I believe tissue culture throws light on how cells function somewhere else. From this, one can make inferences that make it possible to understand better how cells function in their normal environment.

RAISZ: Certainly, as long as you do not try to slide the data over into the *in vitro* situation.

FREMONT-SMITH: There also has been a tendency to say nothing can be learned *in vivo*. I would like to make a prediction, and I would be willing to put it on the record. But 5 or 7 years from now, there will be new information from tissue culture which will have influenced our understanding of cells *in vivo*.

BAUER: What is so special about tissue culture? Is it not true of all techniques, that one must simplify and that one must take into account a system which one is not studying? What is the difference between tissue culture and cytoanalysis? One has to introduce modification in all techniques.

FREMONT-SMITH: These are all model systems, are they not? And none of our histology tells us what the living cell is, because all of the cells are dead. I think it is worth reminding those who are embedded in histology, which is an essential feature of our understanding, that they are not looking at living cells; they are making implications. The only place one can see living cells is in tissue culture, and these cells are not the same; they are in a different environment from those *in vivo*. It is this interaction balance, however, which I am sure will throw light on many fields.

I think that the reaction one gets—I have seen it again and again in these conferences over 30 years—is a great reluctance to accept data coming from another technique or another field. Even the introduction of a new stain is very disturbing to histologists at times, until it becomes more standard. What I am working for, and one of the main purposes of our conference, is to see what we can each learn from the other's techniques, not what we can throw out.

TALMAGE: If one works with an isolated system such as tissue culture, the tendency is to explain the entire physiologic process on the basis of the results from the isolated system. In my opinion, one must make a sincere effort to study the entire physiologic process in the intact animal, and be very cautious of those results from isolated *in vitro* systems which appear contradictory or are unexplainable in relation to the physiologic process as seen *in vivo*.

NICHOLS: Let us go on to Dr. Peck, Mr. Chairman.

PRITCHARD: The problem is simply to find out how cells behave in different environments, both *in vitro* and *in vivo*.

PECK: We have been working on a system that has certain similarities to Dr. Holtzer's. We have been able to disperse cells from fetal and newborn rat calvaria using a crude collagenase preparation—incidentally, we cannot do it with trypsin—and have studied the cells from a number of standpoints; most recently, collagen synthesis (refs. 144 and 145). We made no claims that the cells we have isolated are bone cells, for they have not been shown to produce bone *in vitro*, and it is quite likely that we have harvested a heterogeneous cell population. We have been primarily interested in the relationship between cell proliferation, cell density, and responsiveness to humoral agents, in particular, ascorbic acid and parathyroid hormone.

If we suspend cells in simple incubation medium, we can demonstrate



synthesis of a hydroxyproline-containing protein. The ability of these freshly isolated cells to form peptide-bound hydroxyproline is indicated in figure 72, which depicts the incorporation of radioactive proline into protein, and the appearance of radioactive hydroxyproline in protein, after incubation of cells with labeled proline. The appearance of hydroxyproline, which we can equate with collagen synthesis, increases during the first 6 hours of incubation. After 12 hours, no further hydroxyproline is formed.

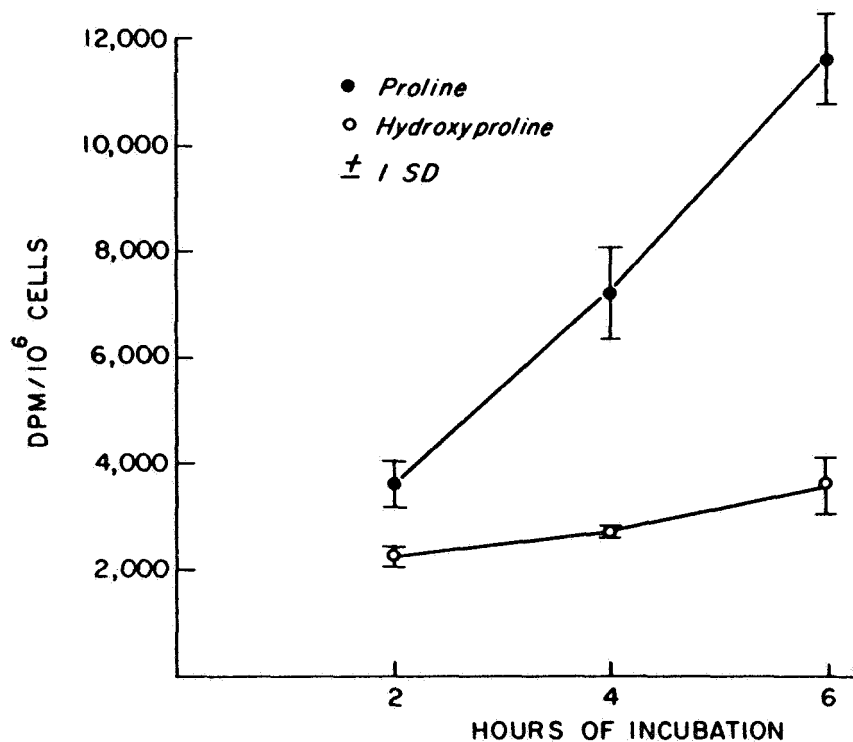


FIGURE 72. Collagen synthesis of freshly isolated bone cells. Freshly isolated cells were incubated in Krebs' Ringer bicarbonate buffer, pH 7.4, gas phase 95 percent O<sub>2</sub>, 5 percent CO<sub>2</sub>, containing L-proline-U-<sup>14</sup>C, dialyzed bovine serum albumin 0.5 percent, glucose 0.11 M, and penicillin and streptomycin 100 units each per milliliter. Each point represents the mean of three flasks. [From ref. 145; reprinted by permission of the publisher.]

FREMONT-SMITH: These are all rat bone?

PECK: These are from the calvaria of rat fetuses that have been dispersed by collagenase. Collagen synthesis by freshly isolated, suspended cells does not respond to a variety of humoral agents,

including insulin, parathyroid hormone, growth hormone and, most disturbingly, ascorbic acid. As you know, ascorbic acid has been found to stimulate collagen formation in a host of *in vitro* and *in vivo* studies with many types of connective tissues. Because of this lack of responsiveness, we decided to maintain the cells under different circumstances.

We dispersed the cells in cell culture on a flat surface, apparently in a fashion similar to the system used by Dr. Holtzer. The cells then go through a period of proliferation. Figure 73 indicates the amount of DNA in the culture with respect to days of culture. It may be seen to increase modestly.

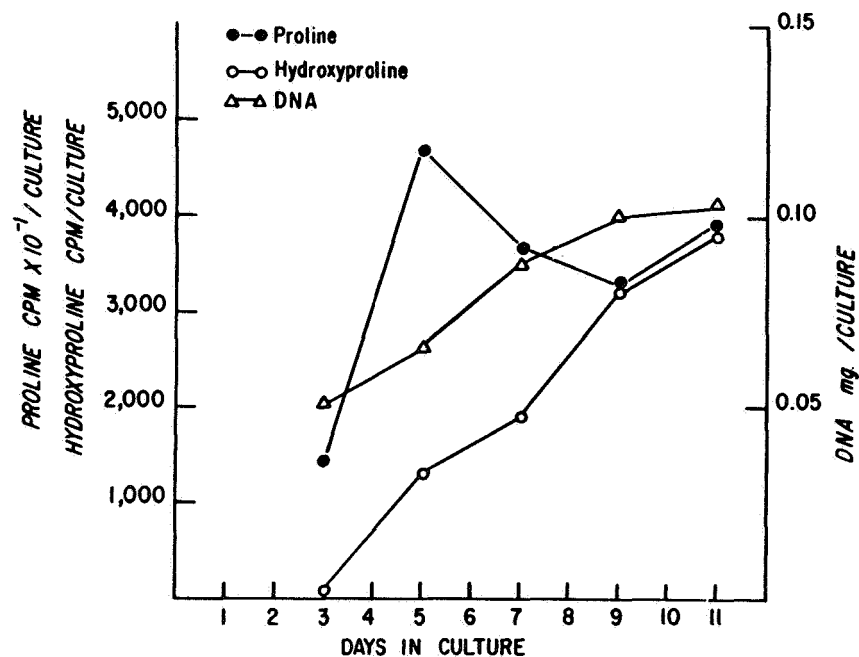


FIGURE 73. Relationships between cell proliferation and appearance of proline and hydroxyproline radioactivity in protein during culture of bone cells on a flat surface. L-proline- $U-^{14}C$  was added to each culture 12 hours before termination, hence data indicate radioactivity incorporated during the preceding 12 hours. [From ref. 145; reprinted by permission of the publisher.]

In this particular series we plated many cells to start with. If one begins with a lot of cells, they do not divide as many times as when one starts with fewer cells. This is because proliferation is limited by the area that contains the cells. Once the cells form a layer that fills the containing area, proliferation slows markedly.

The important point in figure 73 is the appearance of collagen which is represented by the appearance of radioactive hydroxyproline in protein. This was determined by adding  $^{14}\text{C}$ -proline 6 hours before harvesting the culture, so that each point represents the collagen that has been synthesized during the preceding 6 hours. You can see that the rate of collagen synthesis increases with the duration of culture, increases with the accumulation of DNA, and increases with increasing density of the cell population.

If you add ascorbic acid to this cell-culture system, you get a dramatic stimulation of the formation of hydroxyproline-containing protein (fig. 74). We call it collagen formation, at least in the biochemical sense. This is a dose-related phenomenon, as indicated by plotting on semilog paper the concentration of ascorbic acid in  $\mu\text{g}/\text{ml}$  against the appearance of hydroxyproline radioactivity expressed as  $\mu\text{g}$  of DNA in the culture.

URIST: Is the collagenous material uncalcified?

PECK: Yes. We have seen no evidence of bone formation in our

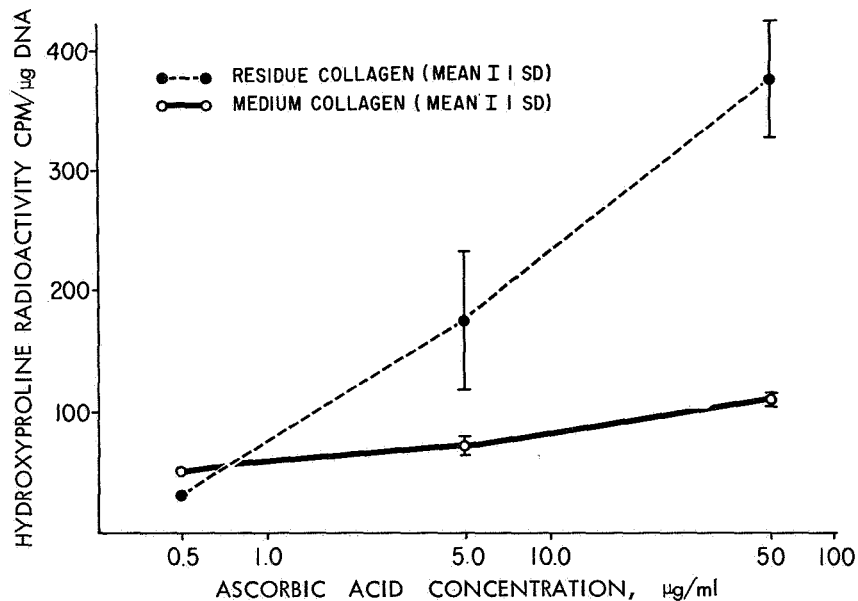


FIGURE 74. Effect of concentration of ascorbic acid on collagen synthesis by bone cells in primary culture. Cultures were incubated in minimal essential medium (Eagle) for 6 hours, in the presence of L-proline- $^{14}\text{C}$ . Residual collagen reflects the hydroxyproline radioactivity of hydrolyzed collagen. Protein was isolated from the medium by Sephadex filtration before hydrolysis. Each point represents the mean of two or three flasks.

cultures. We have seen precipitation of amorphous material, the nature of which remains to be clarified.

URIST: Have you transplanted the tissue back into a living animal?

PECK: We have placed cells in Millipore chambers and implanted them intraperitoneally in growing rats. We get what appears to be, in the Millipore chambers, amorphous material. I discussed this material with Dr. Lent Johnson at the last Gordon Conference.

URIST: Was it calcified?

PECK: Yes. However, none of us could figure out what it was. To reiterate, there is a linear relationship between the log of the concentration of ascorbic acid and the appearance of hydroxyproline (fig. 74). Note that the lower extremity of this curve represents a concentration of 0.5  $\mu\text{g}/\text{ml}$  of ascorbic acid. Generally, the physiologic range of serum ascorbic acid concentrations in humans is about 25 to 30  $\mu\text{g}/\text{ml}$ .

We were interested in the relationship of this ascorbic acid effect to the duration of treatment. As figure 75 indicates, we can detect changes with respect to stimulation of collagen formation within 30 minutes of the addition of ascorbic acid.

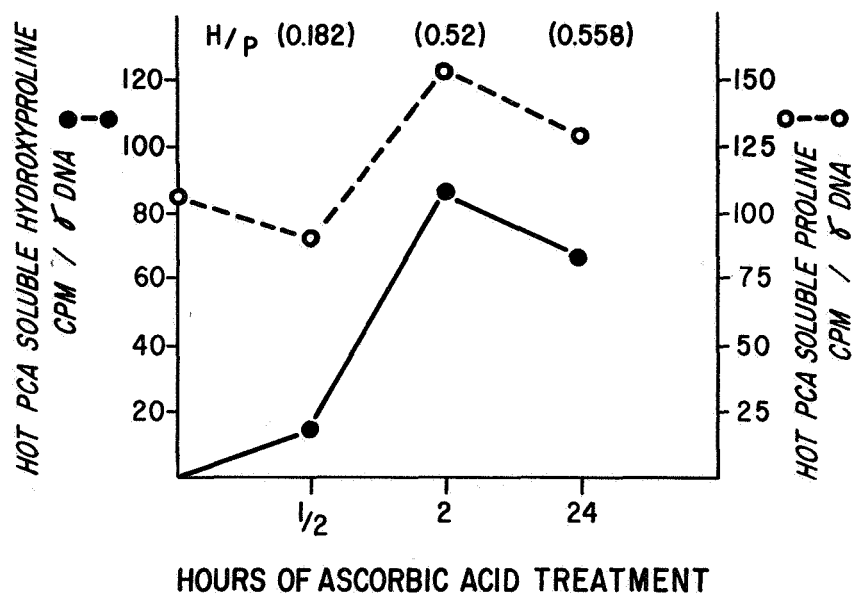


FIGURE 75. Effect of duration of exposure to ascorbic acid on collagen synthesis by bone cells in primary culture; L-proline- $\text{U-}^{14}\text{C}$  was added to each flask 30 minutes before the end of the experiment. Collagen was isolated from the cell layers by two 15-minute extractions with 5 percent perchloric acid at 70° C.

PRITCHARD: I think one could make the point here that ascorbic acid acts on the cell and presumably acts through the DNA-RNA mechanism.

PECK: Initially, it probably acts directly on the process of proline hydroxylation. I would like to think that the stability of the collagen molecule is governed by the hydroxylation of some proline residues and that, in the absence of ascorbic acid, hydroxyproline-poor collagen accumulates. These changes can occur without postulating any fundamental change in the mechanism of the protein synthesis.

I wanted to go into this partly because of the preceding discussion about the value of *in vitro* systems. *In vitro* systems are uniquely suited to deriving strictly biochemical information in a highly controlled environment. This particular system may provide us with some information which will be of value with respect to what is going on *in vivo*, but to draw that conclusion at this point would be totally erroneous.

I will say, in addition, that cultured cells will respond to parathyroid hormone *in vitro* with an inhibition of collagen synthesis which can be reversed by ascorbic acid. At present we are studying growth hormone to see if these cells will respond to that as well.

PRITCHARD: You have a wonderful system which has already given much valuable information.

NICHOLS: Dr. Peck has brought up several important things. One is to point out what happens to cells when they are removed from their site of origin. I wonder, Dr. Peck, if you could tell us about the comparative activity of cells when they are in their normal habitat on surfaces of calvaria, and when they are in suspension?

Modification of all behavior by modification of physical surroundings is extremely important and may well be the control which lies behind differentiation. Incidentally, our *in vitro* systems are really useful here because with them we can examine directly how cells respond to such stimuli.

PECK: One thing we might have done which would have been very helpful was to do essentially what Dr. Holtzer and his colleagues did when they incubated cartilage cells in a button (ref. 146). Our freshly isolated cells were incubated in suspension. We shook them fairly vigorously. It would be interesting to see if these cells maintained more vigorous collagen formation if they were maintained in a button rather than suspended in a simple medium.

The point which remains is obviously a critical one. These cells are derived from tissues that synthesize collagen at a fantastic rate; although we have not done too many studies relating the ability of cells to synthesize collagen with respect to the amount of DNA that

has been released from the bone, our data would at least suggest that the cells are markedly impaired in their ability to synthesize collagen by the process of isolation. This may be open to question, but I think the evidence is quite clear that these are altered cells or that at least some of the cells within the population we isolated are changed.

PRITCHARD: There is another important topic we have not touched yet, the question of induction. I wonder if Dr. Saxén would like to say something about induction systems.

SAXÉN: I will briefly summarize the present situation in the field of embryonic induction and outline our way of thinking. In doing so, there will be no time to present the experimental evidence on which my summary is based, but I will say that most of it is derived from experiments with soft tissues. However, I hope that the general scheme I am going to formulate will be applicable to bone induction and osteogenesis as well, and that both Dr. Holtzer and Dr. Urist will comment on this aspect later.

In my scheme (fig. 76), I have divided the differentiation of a hypothetical cell population into different steps. The responding tissue (which is shaded in the figure) receives an inductive stimulus from the inductor tissue at the beginning of the chain of differentiative events. I do not intend to discuss the specificity of this stimulus, although I may say that I have certain doubts about its specific nature. Let us just call it a stimulus or trigger which initiates the differentiative process in our cell population. If the responding cells are to be capable

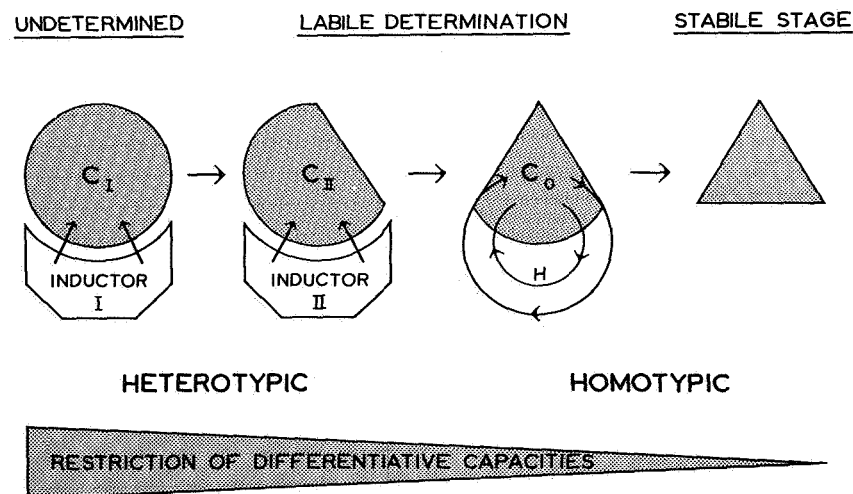


FIGURE 76. Differentiation of a hypothetical cell population.

of responding to this inductive stimulus (I), they should be competent ( $C_I$ )—a characteristic known to disappear during subsequent development.

As a result of this initial inductive stimulus, the responding cells become determined in a certain direction, and consequently lose their competence to respond to similar triggering stimuli. However, this stage of labile determination is followed by subsequent interactive processes. In some model systems at least, the primary stimulus is known to be followed by a second heterotypic induction (II), leading to subsequent determination of the cells. The cells at this stage now display another competence ( $C_{II}$ ), making them capable of responding to the second inductive stimulus.

Subsequent to this (and perhaps even after several heterotypic actions), the responding cells become independent of such epigenetic stimuli, and develop autonomously.

URIST: Please define the term "epigenetic."

SAXÉN: It means "not genetically controlled."

URIST: Thank you.

PRITCHARD: Perhaps I can sum up the discussion so far as follows. From the chemical standpoint, cell differentiation implies change in the kinds or amounts of a cell's synthetic activities. Sooner or later these changes are reflected in the histologic picture—such as mitotic figures, changes of cell shape, size, structure, mobility, change in intercellular matrices, changes in the pattern and organization of the tissues composed of such cells and their matrices.

It is postulated that these changes are brought about by a series of inducing stimuli which evoke responses from competent cells.

The important questions are as follows: What is the nature of the inducing stimuli; where and how do such stimuli act on the cell; how do they select one response, or a particular set of responses, from the cell's repertoire; what is competence in terms of DNA activity; how permanent are the changes induced; what happens when a cell loses one competence and gains another competence?

Inducing stimuli are of many kinds, but now we are particularly interested in the postulated close-range ones which act between one cell and another in its immediate range, which may be like it, or quite different.

As I said, I do not know whether all of these are pertinent to bone induction or morphogenesis, and that is why I would very much like to have comments by Dr. Holtzer and Dr. Urist, who have been studying these problems.

HOLTZER: I think I have spoken enough. I thought that was an excellent summary of the problem.

PRITCHARD: There are still one or two matters to be considered. Some of these concepts are called by different names in the literature; e.g., dependent differentiation, self-differentiation, autoinduction, heteroinduction. Also, there is the question of reversibility, whether one can dedetermine, dedifferentiate.

HOLTZER: No; I disagree. I do not think we should wander all over using such words as they apply to different kinds of cells. I think we should ask Dr. Young or Dr. Peck questions, such as "When, in the life history of bone cells, do they first start making collagen?" or "Do they make collagen during mitosis, G-1, S, or G-2?" and so forth.

YOUNG: I do not believe it is made during S, when the cell is devoted to duplicating its DNA, and I do not think it is made during mitosis. I am sure it is made by osteoblasts during G-1.

PRITCHARD: It must be made by progenitor cells in some stage of their life history.

PECK: Now we are running into the problem we discussed previously of when to call collagen *collagen*. If one waits for morphologically identifiable collagen to be formed, the chances are that the dividing cell will no longer be able to make it. The question is, If there is an arrest in collagen synthesis, where does it occur? Is it arrested at an early stage of synthesis, for example, at the stage of proline hydroxylation, or is it a total arrest, presumably at the genetic level?

YOUNG: My personal bias is that changes in cell specialization are mediated by the selective activation and repression of integrated groups of genes, but I do not think we should go into this now. I would like to hear what Dr. Urist has to say.

OWEN: On the periosteal surface of the shaft of the femur of young rabbits, the osteoblasts line the surface of the bone and behind them are the preosteoblasts, their precursors. In this system, the uptake of glycine per cell in the precursors is about one-tenth the uptake in the osteoblasts.

PRITCHARD: That is a very pertinent observation.

NICHOLS: Dr. Howard Green has some evidence from tissue culture in another system which bears on this point of dividing cells (ref. 147).

HOLTZER: Let us get together. A culture, in which some cells are dividing and some are not, is not a system which permits one to say whether or not a single dividing cell is making collagen. A cell in S is not doing the same thing that a cell in M is doing.

PRITCHARD: This is an academic matter.

HOLTZER: On the contrary, I think these are the only kinds of questions we can approach, otherwise we get back to defining cells by names. You wanted a definition of differentiation, what it means, and of reversibility, and so forth—



PRITCHARD: Call it the collagenoblast or mucopolysaccharidoblast, or something like that.

HOLTZER: Names do not mean very much. We would like to know what a given cell does, and when and under what conditions it does it. We know that during M very little protein and very little RNA is made. What is the nature of such controls? What reactivates transcription and translation after each mitosis? One of the most central problems in all biology is what happens to the cell's synthetic machinery so that it appears to shut down every time the cell goes through mitosis only to be activated in the following G-1. More information on this issue might give us a better basis for words, differentiation, dedifferentiation, induction, and so forth.

PRITCHARD: Perhaps we can leave this topic for the moment and call on Dr. Urist to talk about bone induction.

URIST: The question is, What induces a cell to differentiate and to become an osteoblast? We do not know whether the tissue that Dr. Peck isolated, or the material that Dr. Holtzer explanted, contained induced cells. In the past we have assumed that a cell had been induced at an earlier stage of its development, if after transplantation it demonstrated its capacity to differentiate into an osteoblast. The conditions in tissue culture are less than adequate for osteogenesis, but in the anterior chamber of the eye, conditions are optimum. I will demonstrate osteogenesis induced by germinal cells of articular cartilage with the following figures to be discussed by Dr. Saxén and Dr. Holtzer.

When the cell is at the stage of development of an osteoprogenitor cell, it may be the differentiated form. We cannot tell the difference between a cell that is an osteoprogenitor and one that is a fibroprogenitor by morphologic criteria. We can get some idea by its location and by its rate of mitotic division. We would like to be able to identify an osteoprogenitor cell by its ultrastructure, but Dr. Robinson says that is not yet possible. We do know that because of its proximity to bone, it may have a strong tendency to differentiate into an osteoblast.

The young cells in the germinal layer of the articular cartilage (epiphyseal side) take up tritiated thymidine in larger amounts than other cartilage cells. When a thin slice of the surface of the articular cartilage, a slice containing the flattened chondrocytes, is transplanted in the anterior chamber of the eye as an isograft or as an autograft, the product is either fibrous tissue or induction of new hyaline cartilage (fig. 77(a)).

Another slice (fig. 77(b)), containing the deep or germinal layer of cells, induces bone formation. Before transplantation to the eye, the cartilage cells of the germinal layer were labeled with  $^3\text{H}$ -thymidine, and the cartilage matrix was labeled with  $^3\text{H}$ -glycine by intra-articular

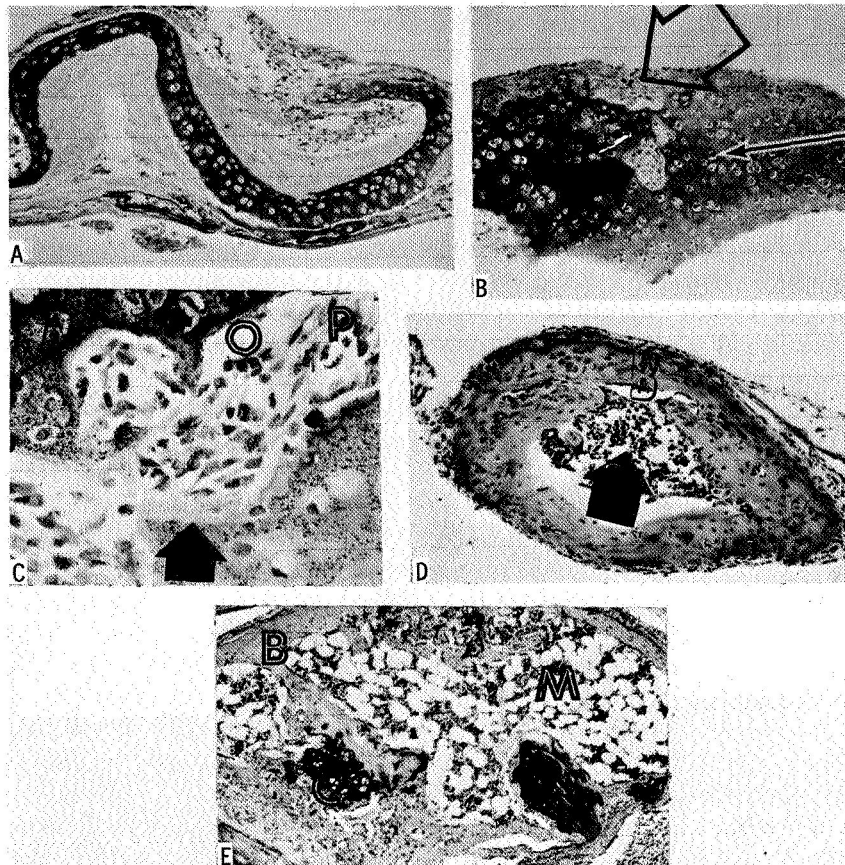


FIGURE 77. Photomicrographs of sections of articular cartilage transferred to the anterior chamber of the eye. PAS-hematoxylin stain.

(a) Thin slice of gliding surface of cartilage, 6 weeks in the eye. Transplant is enveloped in capsule of fibrous connective tissue; neither excavation chamber nor osteogenesis is produced. 30 $\times$ .

(b) Coronal section, full thickness slice of cartilage: labeled chondrocytes (black and white arrow); excavation chamber filled with capillary sprouts, new connective tissue, and a pair of labeled chondrocytes liberated from their capsules and free in center (open arrow); excavation chamber filled with new cells but no labeled chondrocytes (solid arrow). 30 $\times$ .

(c) Autoradiograph: cement line dividing glycine-labeled old cartilage from layer of unlabeled new bone matrix (solid arrow); labeled chondrocytes (open arrow); labeled progenitor cells (P) and osteoblasts (O) suggest that labeled chondrocytes modulate into bone cells. 30 $\times$ .

(d) Ossicle formed from transplant. New lamellar bone (B) surrounds pool of marrow (M) and contains islands of unresorbed cartilage (C). 60 $\times$ .

(e) Complete replacement of donor tissue. Ossicle composed of compact cortical bone (B) and healthy hematopoietic marrow (arrow). 60 $\times$ .

injections. The labeled cartilage was transferred to the anterior chamber of the eye of another rat of the same inbred strain for a period of 3 weeks. There was no translocation of tritiated glycine from the matrix of the donor to the cytoplasm of the new cells of the host. Figure 77(c) is an autoradiograph of an area of osteogenesis in an excavation chamber in an isograft of articular cartilage.

By means of tritiated thymidine labeling experiments, one can see that the cells dissolve the capsule and surrounding matrix and undergo mitotic division. The first mitotic division produces connective tissue cells that look like mesenchymal cells. Six weeks after the operation an ossicle is formed (fig. 77(d)) and there is complete replacement of all of the donor tissue (fig. 77(e)).

The second, third, or fourth mitotic division—we do not know how many in 15 days—occurs in such a way that the capillary growing into the transplant interacts with the progeny of these germinal cells that have dedifferentiated or modulated, depending upon how one wants to look at it, and the interaction, or what Paul Weiss calls the *swarming* of these cells in two different locations, results in what appears to be a progenitor cell. We say this is a progenitor cell because its rate of uptake of tritiated thymidine is greater than that of any other cell in the area. It is by this criterion and by none other that we call it a progenitor cell. I am not even saying what kind, whether it is osteoblast, chondroblast, hematoblast, or something else.

PRITCHARD: You could call it a thymidine cell.

URIST: You could call it a thymidine-labeled connective tissue cell. The progenitor cells differentiate into osteoblasts. The interaction of the progeny of the cells from two sources produced bone. The progeny of one population of cells induced the progeny of the other to become osteoblasts and to make bone. The cells were not induced to make more cartilage in the new environment in which their progeny proliferated; instead, the product was new bone.

Figure 78 is a diagrammatic representation of the ordered sequence of events in the induction system for bone from articular cartilage. During the first 10 days after transplantation, the germinal cells of articular cartilage lyse the intercellular substance and divide mitotically to produce inducing cells. Swarming occurs between 10 and 15 days, and a large number of ingrowing perivascular inducible connective tissue cells of the host interact with a relatively small number of progeny of donor cells. Between 15 and 20 days, the donor tissue develops excavation chambers filled with sprouting capillaries and proliferating young connective tissue cells. Microscopically, the progeny of the donor and the host are identical, but functionally they are different insofar as some have been induced to differentiate or specialize as osteoblasts, chondroblasts, hematocytoblasts, or fibroblasts. Some

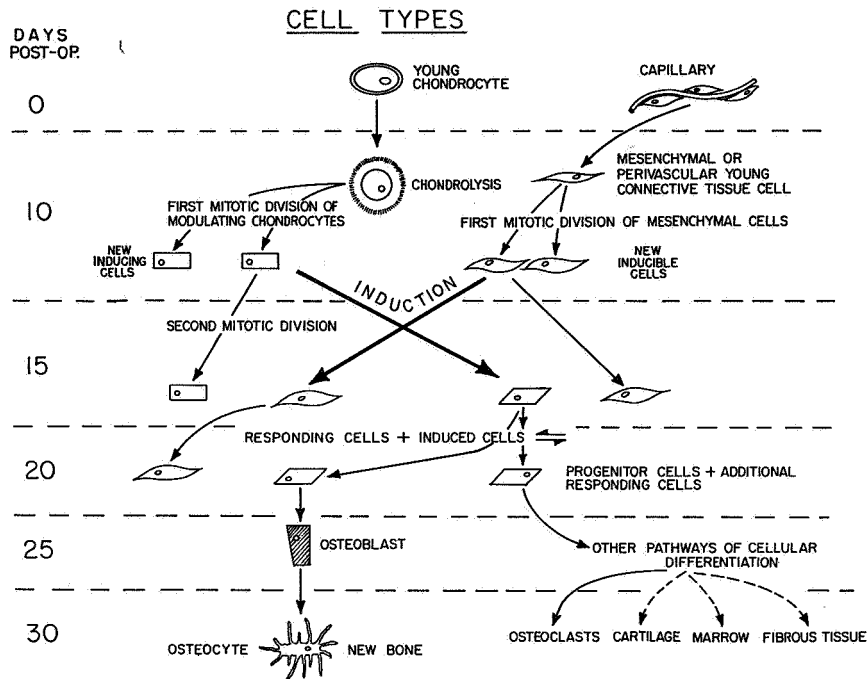


FIGURE 78. Ordered sequence of events in the induction system for bone from articular cartilage.

divide and produce more responding or undifferentiated connective tissue cells. Between 20 and 25 days, the induced cells differentiate into osteoblasts; between 25 and 30 days, the new bone containing osteocytes is deposited in one location while new marrow, fibrous tissue, and cartilage are deposited in another. Osteoclasts appeared only in association with bone tissue and with the onset of remodeling of the initial deposits.

The questions, Dr. Saxén, are these: What was transferred from one cell population to another? Was anything transferred? Was the inducer something that arose intercellularly as a result of this change in environment, or was some chemical inducer transferred from the donor to host progeny? Can you explain this reaction with the aid of your hypothesis?

SAXÉN: I am afraid, Dr. Urist, I cannot answer your questions. I pointed out that we do know that there are what I call epigenetic factors, but we do not know anything, or very little, about their chemical nature and their mode of action. For instance, you mentioned the anterior chamber of the eye. Kidney induction, as I showed, takes place under the very same conditions without its specific inducer. So

now there are people who are inclined to speak of "conditions" rather than of specific inducers.

So the only thing I can say is that these cells have been exposed to different environments. Whatever it is in these environments that causes this determination is not known.

I would like to know what Dr. Holtzer has to say about this; he is more familiar with these cells.

URIST: Dr. Holtzer, in the embryonic notochordal tissue-spinal cord induction system for cartilage, is there evidence that a substance is transferred from one cell to another?

HOLTZER: We have no evidence.

URIST: Is there evidence of a diffusible substance?

HOLTZER: Absolutely none.

URIST: In the experiments of Lash et al. (ref. 148), did cartilage induction occur from a substance that diffused across a Millipore membrane?

HOLTZER: There is an interaction. We never pretended that we had a molecule that we could name. We are asking a kind of question that is experimentally beyond our capacity, certainly my capacity right now, to analyze. That is why I prefer to refocus some of our questions in differentiation. We are all looking for little devils to send a cell in this direction, send a cell in that direction, and that's the end of it. On the contrary, there is excellent evidence in the case of bone that it is not that simple; that there are no demons that open trapdoors and let a message come through and that is the end of the interaction.

Therefore, why belabor something which people studying bone have demonstrated year after year? That is to say that there is continual interaction and that there are, in fact, alterations in the states of a cell. We define, by specifying the activity, what we mean by states of alteration.

The question you are asking, as far as I am concerned right now, is a rhetorical one because nobody has any evidence that an information-bearing molecule comes from one source, goes into another cell, and diverts that cell to another task.

PRITCHARD: In some setups, surely it does; you are a male and I am a male because at a certain stage in our development a certain hormone was produced by—

HOLTZER: Remember, I said "an information-bearing molecule" which changes the fate of a cell. By and large, hormones and other exogenous molecules accelerate or dampen an activity; they permit cells to express a capacity that was built into them by their previous history. To my knowledge there is no conclusive evidence that the known hormones establish that capacity. A hormone acts on a target cell. But the inductive influence, which initially guided the differentia-

tion of that target cell, was not that particular hormone. Hormones act on programmed cells, not naive cells. Hormones select preset programs. The central problem of differentiation is the nature of the influences that establish the programs. At this point, with the possible exception of vitamin A, there is no clear-cut evidence of an "information-rich" molecule from one cell entering another and thereby altering the latter's fate.

PRITCHARD: A hormone is an information-bearing molecule.

HOLTZER: Does it go inside the second cell?

PRITCHARD: Yes.

HOLTZER: I am sorry; you might be right.

PRITCHARD: You see, once you label these things—

HOLTZER: I am sorry—I do not accept any of that work.

SAXÉN: I would like to add something to this matter of specific action. In quite another system, the developing central nervous system, it has been shown that factors such as CO<sub>2</sub> shock, a slight change in the pH of the environment, and things like that can trigger this chain of events. In these situations we certainly are not dealing with an information molecule, and that is one reason why I said I have my doubts about the specificity of this approach.

RAISZ: Dr. Urist, have you tested any changes in the microenvironment in the eye for the ability to induce bone; for example, calcium changes or something of that sort?

URIST: Some years ago in Dr. McLean's laboratory, Heinen (ref. 149) put rats on a phosphate-deficient diet, made them rachitic, cultured the bone, and transplanted the culture to the anterior chamber of the eye. The new bone was phosphate-deficient and vitamin D-deficient rachitic bone or osteoid tissue. The culture differentiated into bone, but the matrix did not calcify. Bone induction can take place even under conditions that are not conducive to calcification of the matrix.

RAISZ: That is what I was asking you: whether there were times that one could get cartilage or bone under some conditions of changing environment.

URIST: Yes; we will try to get to that question when we present a few more experimental observations.

We implanted a segment of HCl-decalcified lyophilized cortical bone into a pouch in the anterior abdominal wall, or the quadriceps muscle, and observed bone induction in excavation chambers inside the old matrix. Cartilage induction occurred inside old vascular channels.

FREMONT-SMITH: What was the origin of the decalcified bone? Also rabbit?

URIST: Yes, rabbit; we employed bone from the same or another individual.

FREMONT-SMITH: Is the effect not species specific?

URIST: No; it is not. We have also observed what Dr. Saxén calls heterogenous induction with implants of bovine and human matrix in rabbits (ref. 150), but the inflammatory reaction is high, the percent positive results low, and the yield of bone is very, very small.

I will now show some examples of bone induction by heterogenous, dead, decalcified bone matrix. Figure 79(a) is an implant of bovine decalcified bone matrix in the anterior abdominal wall of a rabbit. The implant is enclosed in an envelope of plasma cells, lymphocytes, reticulocytes, and foreign body giant cells. Figure 79(b) illustrates scanty deposits of new bone on the surfaces of an implant of dead, decalcified bovine matrix, inside the envelope of inflammatory tissue in the anterior abdominal wall of a rabbit, 12 weeks after the operation. Figure 79(c) shows the implant of bovine dead, decalcified bone matrix in the anterior abdominal wall of a rat, 12 weeks after the operation. The rat has a high propensity for formation of cartilage on the walls of vascular channels in the old matrix. These cells differentiate from histiocytes that wander into and repopulate nearly every crevice and space with new cells. In the process of cartilage induction, there is little or no resorption of the inducing surface.

It is necessary to emphasize, however, that while the inducing material is heterogenous in origin, in these systems both the inducing cells and the induced cells come from the host bed. For this reason, I refer to an article on bone formation by autoinduction (ref. 151).

We have implanted decalcified, lyophilized muscles, tendon, kidney, and other tissues into the anterior abdominal wall, and the results were negative over a period of 8 weeks. It is necessary to extend this experiment to 3 to 6 months; bone formation does not appear in injured tendons until after 3 months. Cartilage, however, is different; decalcified, lyophilized costal cartilage will induce bone formation with an incidence of over 60 percent positive results.

PRITCHARD: Dead cartilage and muscle will also show this, and there is a comparable delay before new bone appears.

URIST: Bone induction was influenced by the effects of the acid that was used to decalcify the matrix. Nitrous and nitric acids, which deaminate protein, prevent bone induction; HCl, EDTA, and formic-citric acid do not alter the matrix in a comparable way and do not inhibit bone induction. Nitric acid-decalcified matrix produces an extensive foreign body giant cell response.

Figure 80(a) illustrates a deposit of new bone on the walls of an excavation chamber in an implant of homogenous dead, decalcified bone matrix, 4 weeks after the operation in a rabbit. Figure 80(b) shows deposit of new bone and remnants of an implant of old dead, homogenous, decalcified bone matrix in a rabbit 8 weeks after the

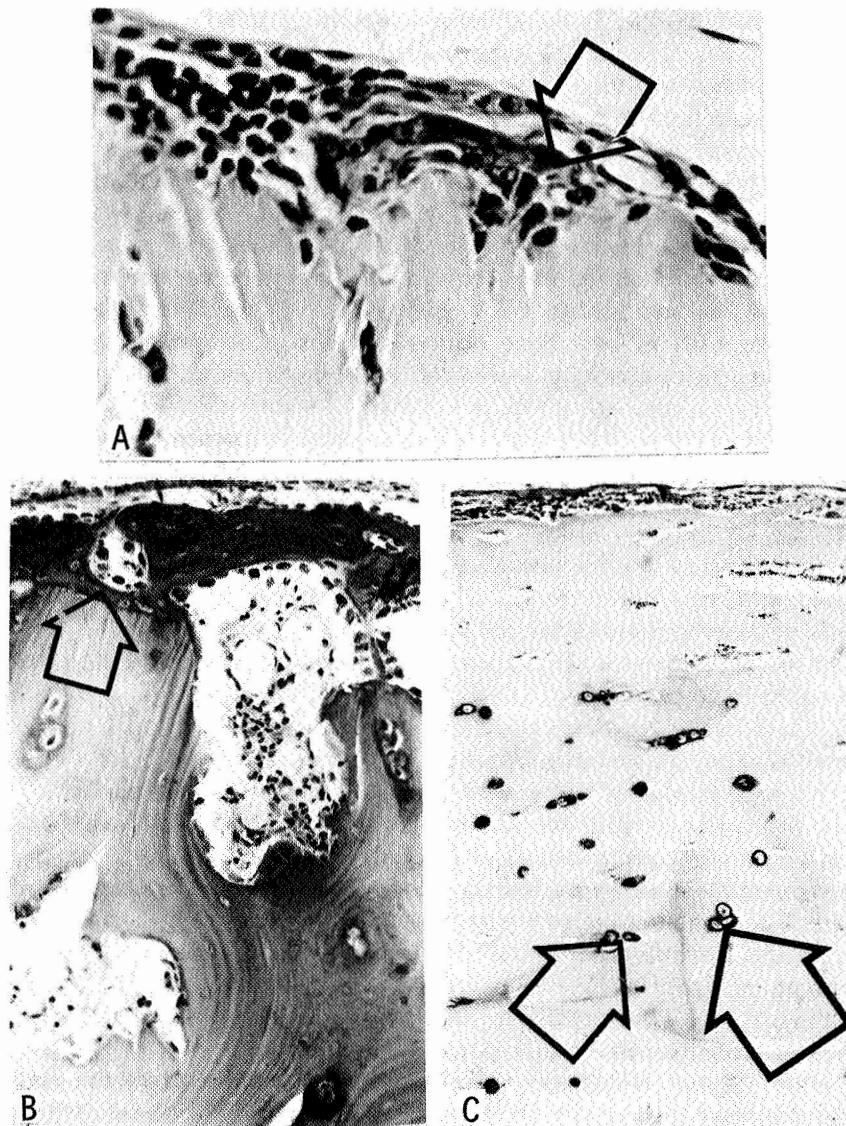


FIGURE 79. Photomicrographs of bovine decalcified bone matrix implants in the anterior abdominal wall of the following:

(a) *Rabbit*—the implant is enclosed in an envelope of plasma cells, lymphocytes, reticulocytes, and foreign-body giant cells (arrow).

(b) *Rabbit*—scanty deposits of new bone (arrow) on surface of implant inside envelope of inflammatory tissue.

(c) *Rat*—cartilage on walls of vascular channels in the old matrix (arrows).



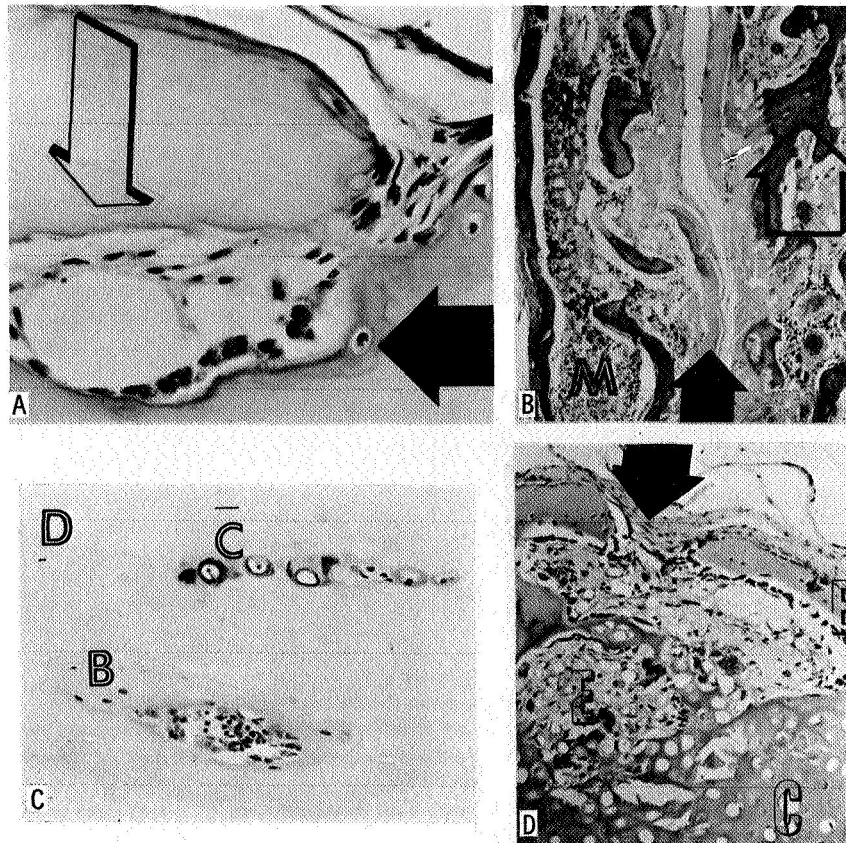


FIGURE 80. Photomicrographs of homogenous, dead, decalcified bone matrix implants in the anterior abdominal wall of a rabbit. HEA stain.

(a) New bone deposit on walls of excavation chamber; osteocyte (solid arrow) and empty lacuna (open arrow) in substance of the old matrix.

(b) New bone deposit (open arrow); remnants of old dead bone matrix (solid arrow); bone marrow (*M*).

(c) Decalcified, lyophilized bone matrix (*D*); chondrogenesis (*C*) in an old vascular channel; osteogenesis (*B*) in new excavation chamber.

(d) Decalcified, lyophilized costal cartilage; excavation chamber (*E*) filled with proliferating connective tissue in old acellular dead cartilage (*C*), deposit of new bone (*B*) and implant enclosed in a sheath of fibrous connective tissue (arrow).

operation. Figure 80(c) is an implant of HCl-decalcified, lyophilized bone matrix showing chondrogenesis in an old vascular channel and osteogenesis in a new excavation chamber. Figure 80(d) is a section of lyophilized, decalcified, homogenous costal cartilage, 8 weeks after implantation in the anterior abdominal wall of a young rabbit. The excavation chamber is filled with proliferating connective tissue

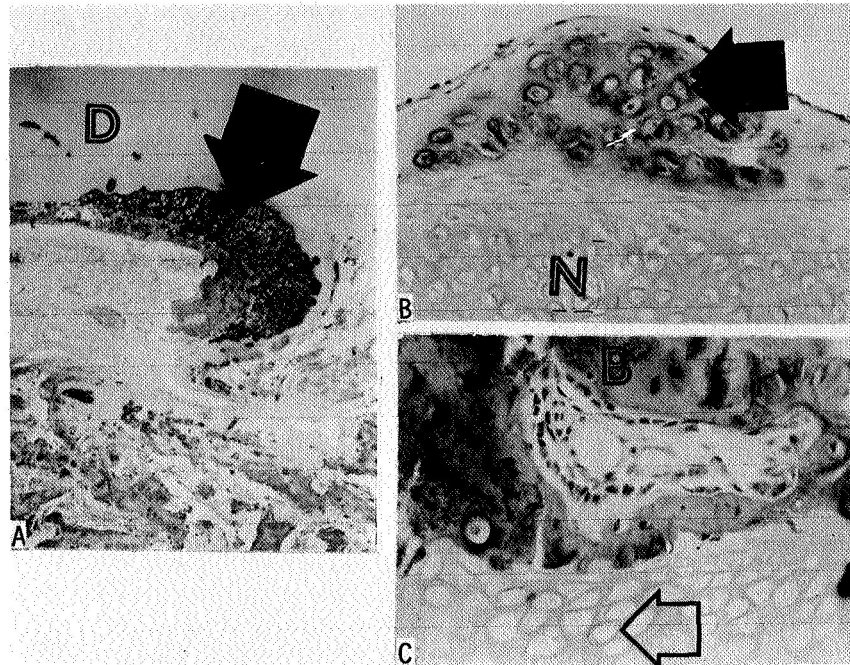


FIGURE 81. Photomicrographs of implants in the anterior abdominal wall.

(a) Island of new cartilage (arrow) on inner wall of decalcified homogenous bone matrix (D); new bone (bottom) and old dead matrix (top) surround area of chondrogenesis.

(b) Homogenous dead, decalcified, lyophilized nasal cartilage (N); induction of new cartilage formation (arrow).

(c) Homogenous dead, decalcified, lyophilized nasal cartilage (arrow); formation of excavation chamber and induction of new bone formation (B).

in old acellular dead cartilage, there is deposit of new bone, and the implant is enclosed in a sheath of fibrous connective tissue.

Figure 81(a) shows an island of formation of new cartilage on the inner wall of an implant of decalcified, homogenous bone matrix 6 weeks after the operation. Such masses of cartilage are later resorbed and replaced by new bone through typical endochondral ossification. New bone and old dead matrix surround the area of chondrogenesis. Figure 81(b) is an implant of homogenous, dead, decalcified, lyophilized nasal cartilage of a rat implanted in the anterior abdominal wall of a rat showing induction of new cartilage formation. Figure 81(c) is an implant of homogenous, dead, decalcified, lyophilized nasal cartilage showing formation of an excavation chamber and induction of new bone formation 6 weeks after the implant operation.

BÉLANGER: Were all of these made at the same time approximately?

URIST: The implants were excised at intervals of 4, 6, 8, and 12 weeks after the operation.

HOLTZER: Cells are invading the matrix. Can you say anything about them?

URIST: Yes; leukocytes, histiocytes, foreign body giant cells, sprouting capillaries, and perivascular connective tissue cells pass in review through the tissue in sequence at various times in various proportions, in various sites in the implant. The implant is at first unoccupied territory that gradually becomes repopulated with cells.

Figure 82(a) is homogenous, decalcified bone from a Belgian rabbit implanted in the anterior abdominal wall of a New Zealand rabbit without lyophilization, 4 weeks after the operation. Note the infiltration of inflammatory connective tissue cells and the dissolution of the bone matrix, but no new bone formation. Bone induction is retarded or suppressed by inflammation either from an immune response or from sepsis. Figure 82(b) is a roentgenograph of the anterior abdominal wall of a rabbit containing eight Millipore chambers. Four chambers contain transplants of fresh, autogenous, cancellous, viable bone tissue, and four contain 0.6 N HCl-decalcified dead bone matrix, 4 weeks after the operation. Bone tissue was formed inside, never outside, the membrane of the Millipore chamber containing viable bone tissue. There was no bone formation either inside or outside the chambers containing dead, decalcified bone matrix. When there was a defect in the Millipore membrane, the host cells were found in a stream growing into the chamber; these cells were able to gain contact with the dead matrix to set up an induction system for osteogenesis.

SAXÉN: The grafts are not rejected when you use a heterograft?

URIST: The plasma cell-reticulocyte reaction around the implant suggests that it is rejected. But it cannot be discarded. Instead, it is encapsulated in an envelope of inflammatory connective tissue cells. It is understandable that prolonged inflammation would inhibit cellular differentiation of cartilage and bone. Prolonged inflammation should induce differentiation of leukocytes and macrophages; cells specializing in phagocytosis, not in production of intercellular substances.

Except that cell specialization may begin in the aftermath of sterile inflammation at about 21 days, it is difficult to understand how chondrogenesis is induced in the blind end of an old vascular channel, or how osteogenesis is induced on the walls of an excavation chamber in decalcified matrix. In the case of cartilage, the surface of old matrix appears to be unaltered morphologically. On a molecular level, of course, it is possible to envisage a mechanism whereby the old matrix

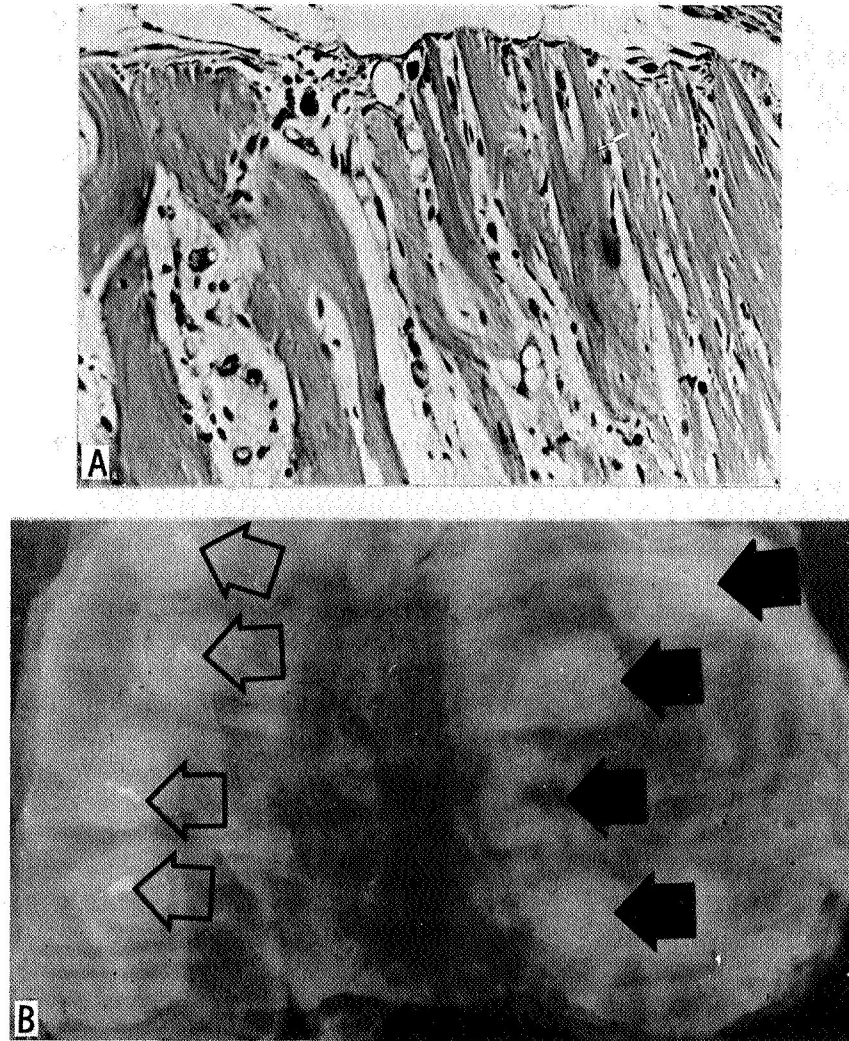


FIGURE 82. Implants in anterior abdominal wall of rabbit.

(a) Photomicrograph of inflammatory response from heterograft.

(b) Roentgenograph of abdominal wall with Millipore chambers *in situ*; fresh autogenous, cancellous, viable bone (open arrow); decalcified, dead bone matrix (solid arrow).

may transfer a template of protein structure onto the plasma membrane of young connective tissue cells; this can be transferred to the ribosomal membranes and then relayed to the regulator genes to produce cellular differentiation, either of a chondroblast or an osteoblast. These ideas, however, are conjectural and do not take into consideration many other factors in the host bed that are unspecific or unknown in nature.

PRITCHARD: I would like to show a figure of an implant of dead muscle that is being invaded by cells which turn into cartilage cells in an almost identical manner (fig. 83).

CURREY: Was this piece of muscle frozen?

PRITCHARD: No; it was fixed in acetone, but we are evidently dealing with a similar phenomenon.

URIST: The phenomenon is similar but not the same.

SAXÉN: Dr. Urist, do you have any information on the distance; that is, how far from the graft will new bone be formed?

URIST: The first row of cells in contact with the old decalcified matrix may differentiate into a row of osteoblasts, but we have seen the second row of cells produce bone without direct contact with the implant.

SAXÉN: How far from the graft is this new activity seen? If there is a transmission of something, there should be a maximal distance, which is quite well known in different induction systems.

URIST: The cells that grow in with a capillary sprout and produce an excavation chamber are the same cells that become osteoprogenitor cells, osteoblasts, and new bone. The distance across the excavation chambers produced by these cells is approximately 100 to 150 microns

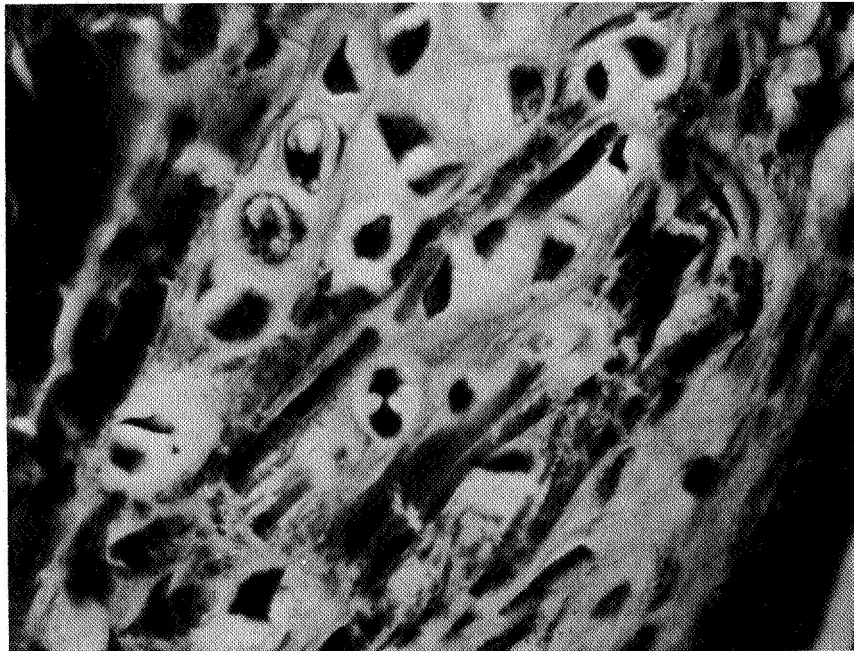


FIGURE 83. Photomicrograph showing rows of cartilage cells between fibers of acetone-fixed tongue muscle of the rabbit implanted beneath the kidney capsule. 500 $\times$ .

in diameter. Does a chemical substance diffuse from matrix into the cytoplasm of the matrix-resorbing cells? Perhaps later I will present some experiments designed to potentiate, to degrade, or to inactivate the matrix that produced a bone induction system.

It should be noted that extraction or partial denaturation of the decalcified matrix with alcohol, strong salt solution, ether, acetone, and detergents did not retard bone induction. Autogenous bone matrix was not better than homogenous bone matrix for bone induction when implanted after devitalization, decalcification, denaturation, and extraction by these agents. Heating the implant to 100° C gelatinized the matrix and, like nitric acid and FDNB, prevented bone induction. Thus, although the structure of the protein of the bone matrix is an important factor, it is impossible to assume that something diffusible was transferred to ingrowing cells.

PRITCHARD: One can approach the problem in part by morphology. Whatever material is excavated, there is always a chance that the mesenchyme that goes in will make bone. It is a common phenomenon. The tunneling is done in the hard material; then the cells differentiate on the walls of the tunnels. Because these cells, sitting on the walls of the tunnel, are in direct contact with the bone, the distance is virtually nil. That was the question Dr. Saxén was asking.

URIST: The line of contact between the decalcified material and the cell might consist of one cell or one layer of cells; but the influence of the substance of the old bone matrix is upon a new population of cells, many of which do not appear to have direct contact with the implanted material. It is possible that a relay system is involved; the first layer of responding cells may become a layer of inducing cells, and layer by layer the inducer is transmitted from one cell to the next until the excavation chamber is filled with a laminated mass of bone except for a single blood vessel in the center.

PRITCHARD: Which one turned into the osteoblast, the one on the surface or the one in the middle?

URIST: Sometimes we see osteoblasts developing in an area separated from the old matrix by several layers of cells.

PRITCHARD: I have not seen this.

URIST: We do not see osteogenesis only in lines of contact with the old decalcified matrix; two or three layers of cells may be involved in the process in some areas of the resorbing implant.

I will try to present some experiments on matrix treated with blocking reagents and enzymes, designed to show whether or not something is transferred.

The question that we have before us is, What is the arrangement of the young connective tissue cells when a bone-induction system



begins in the interior of an implant of HCl-decalcified, lyophilized bone matrix, 21 days after the operation? Figure 84(a) is a section showing the old matrix, or inducing surface; the layer of young connective tissue cells, a mixture of inducing cells and reponding cells, in contact with the inducing surface; layers of proliferating fibrous connective tissue cells, or pool of new responding cells; and the mass of loose fibrous connective tissue and inflammatory cells.

Figure 84(b) is a higher magnification of the mixture of inducing and responding cells; the area indicated by (B) is the first layer in contact with the inducing surface, or old matrix. There is densely staining basophilic cytoplasm of some of the cells, resembling osteoblasts. Others with clear cytoplasm and dense nuclei resemble osteoprogenitor cells. Figure 84(c) shows connective tissue in the center of the implant of decalcified matrix in a rabbit given an arterial injection of india ink to label the macrophages. Deposits of new cartilage are seen in the old vascular channels. A higher magnification

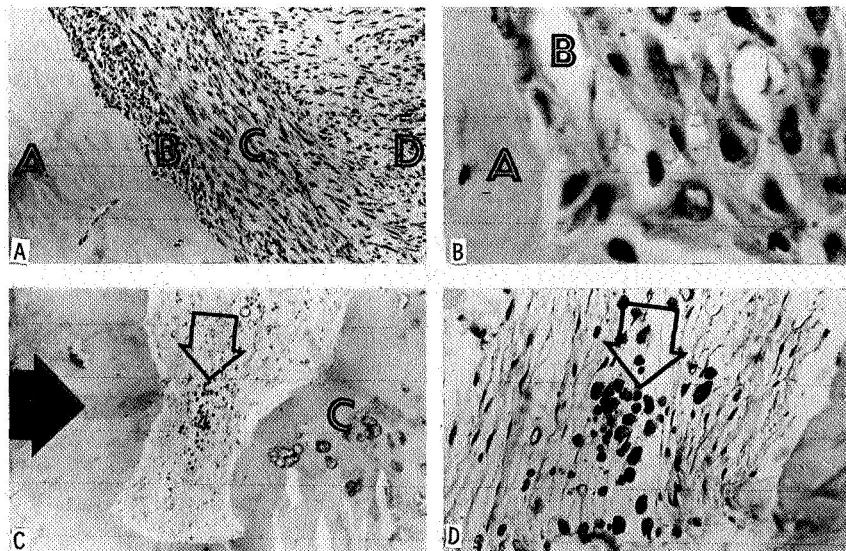


FIGURE 84. Photomicrographs of sections of HCl-decalcified, lyophilized bone matrix implants 21 days after the operation.

(a) Old matrix (A), layer of young connective tissue cells (B), layers of proliferating fibrous connective tissue cells (C), and loose fibrous connective tissue and inflammatory cells (D).

(b) Old matrix (A); first layer in contact with inducing surface (B).

(c) Center of decalcified matrix (solid arrow) after injection of india ink to label macrophages (open arrow). Note deposits of new cartilage (C) in old vascular channels.

(d) Location of macrophages filled with phagocytosed particles of india ink; none was found in macrophages in contact with old decalcified matrix.

of this area is shown in figure 84(d) to demonstrate the location of macrophages filled with phagocytosed particles of india ink; none was found in macrophages in contact with the old decalcified bone matrix.

In summary, the observations on early stages of bone induction suggest that the young connective tissue cells in contact with the bone matrix are several layers in thickness. During the period from 19 to 23 days after the operation, the young proliferating cells may move on and off the inducing surface, interact and swarm. At approximately 24 days a layer of osteoblasts appears, and bone is formed on the inducing surface of old matrix. After that, the layer of new bone may become the inducing surface, and the next layer of osteoblast may become the inducing cells, and so on.

To determine the sequence of events and the mitotic activity of the layer of inducing cells in contact with bone matrix, the host was injected with  $^3\text{H}$ -thymidine and the implant excised at 1 and 5 days later during the 19- to 23-day period of bone induction. Autoradiographs demonstrated that the cells with the highest percentage of labeled nuclei were located along the inducing surface of old matrix and near areas of osteogenesis. The cells with the lowest percentage of labeled nuclei were located in areas of resorption and phagocytosis and some distance away from areas of osteogenesis. The percentage of labeled cells was also low in areas of chondrogenesis, possibly because the rate of mitosis of the cartilage cells is relatively low compared with that of osteoprogenitor cells.



## SYSTEM DISEASES OF BONE

### Discussion Leader:

DR. GEORGE NICHOLS, JR.

NICHOLS: There are three reasons for including a discussion on "System Diseases of Bone" in a symposium of this type.

The first reason is that clinical bone disease occurs or will occur in the majority of us by the time we leave this planet. Osteoporosis, for example, can be shown to be present—even though it may be asymptomatic—in close to 50 percent of all women over 60 and is almost equally common in men, although it appears rather late in life. Since the origins and treatment of these diseases are largely unknown, there are good reasons for attacking these problems from a purely medical point of view. The clinicians badly need the help of their colleagues in basic science in finding the new answers which they seek.

A second reason is that this discussion is of use to those of us who are basic scientists if it brings to our attention the natural experiment provided by disease. Such experiments have told us more than once what questions to ask. Sometimes we have found the answer by turning to man, sometimes by turning back to the animal with new insight. Later I will present some examples which will perhaps illustrate this point.

Third, there are reasons, well illustrated by the discussions of earlier sessions, to think that we are at the beginning of a new era in our understanding of bone and its diseases. Those of us who are physicians will remember that bone disease was first described in terms of gross structural disturbance. This began, probably, as far back as the Egyptians and has gone on ever since. More recently, bone disease was studied in terms of mineral metabolism because it was easier to correlate the nutritional aspects of mineral metabolism with bone disease than anything else that could be measured at that time. So we went through an era dominated by Albright, in which external balances of calcium, phosphorus, and nitrogen were cor-

related with various forms of bone disease. We are now reaching a point where we can start to think about bone disease in yet another way, in terms of cell or tissue biology; it is to this aspect that I will address my remarks.

Bone disease, approached at the level of cell biology, can be thought about in quite simple terms. There are actually only two sets of processes going on: accretion and resorption. Once the details of these processes are known, including their initiation and control, it is possible to describe the phenomena of embryogenesis, of growth or remodeling, and perhaps of calcium and phosphorus homeostasis in terms of these two systems. By the same token, we will be able to describe bone disease in terms of the derangement of these two processes and the balance between them.

I will speak almost entirely about matrix, partly because one can deal with only one thing at a time and partly because matrix appears as the primary material which determines form; the chemical characteristics of the matrix, once they have been laid down, serve to attract the necessary mineral to provide rigidity. Although mineral metabolism may affect the processes of resorption and secretion of matrix in some situations, in general it tends to be a follower rather than a leader.

The first step in developing these ideas is to list those things we wish to know in order to look at bone disease. We need to understand the nature and the sequence of the steps involved in biosynthesis of matrix, the substrate requirements and rate of each. Similarly, we must know how resorption takes place and what steps in the process are rate limiting. Finally, we will need to know what controls exist for each overall process and how they work. This category includes not only such things as substrate availability and hormones but also a host of other factors, which may well be the most important of all, such as responses to local stimuli. This information will provide answers to such intriguing questions as "What tells bone cells that a fracture has occurred and callus formation is needed?"

Figure 85, which I prepared for another symposium a year ago, summarizes the state of our knowledge about bone-cell metabolic systems and provides us with ideas of where controls might be applied. The information on which this diagram is based has been provided by a number of sources, some dealing with bone and some with other connective tissues. As can be seen, we have quite a bit of biochemical information about bone cells. There are transport systems for glucose and amino acids (shown by the circle at the left of the cell) which get the raw materials in. We know that amino acids are concentrated in the cell either by a transport system or by synthesis from glucose as a precursor. It has also been shown that these amino acids are activated,

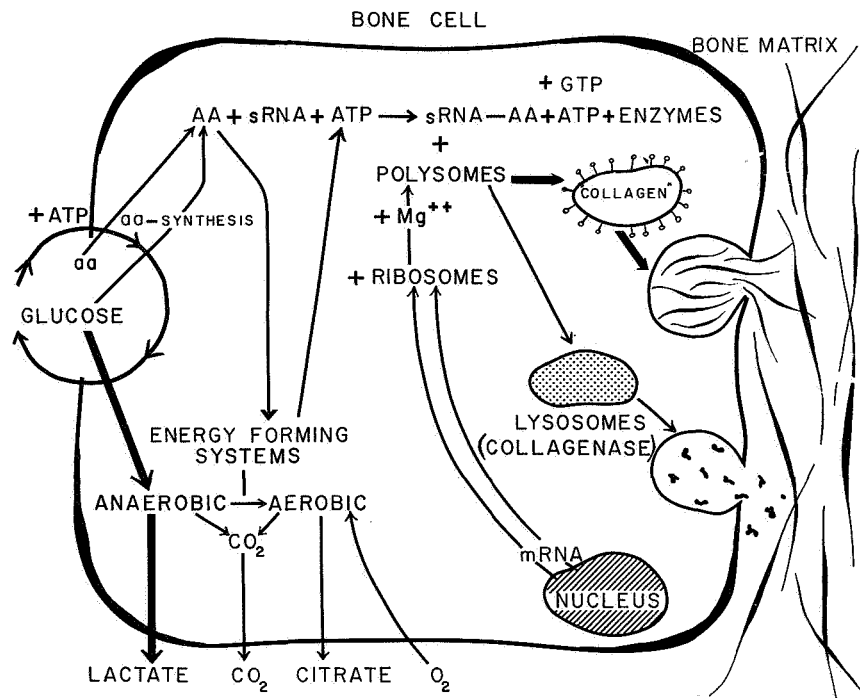


FIGURE 85. Bone-cell metabolic systems.

tied onto their appropriate transfer RNA's, and assembled, presumably by a ribosomal mechanism, into collagen which is then stored in vesicles and finally extruded into the extracellular phase. The rate of each of these steps and of the overall process can be measured. A second arrow emerging from the protein synthetic scheme, and leading to lysosomes and collagenase, has been included to remind us that these factors are present and involved in resorption. These collagenase-containing bodies have been shown as storage vesicles which also extrude their contents extracellularly to indicate that the collagenase must work outside rather than inside the cell. Whether two separate ribosomal paths exist (in the same or different cells) for these two processes is not known, but the presumption can be made that the information for the biosynthesis of each flows from the nucleus via messenger RNA.

Energy to drive these systems is needed; so ATP is formed by a breakdown of glucose, partly by glycolysis and production of lactic acid and partly by an oxygen utilizing system with the production of CO<sub>2</sub> and, in this particular cell, the release of citric acid as well. The relation of this acid production to bone-mineral mobilization and hence resorption has been suggested so often that it need not be reviewed here.

One of the conveniences of this scheme is that it permits potential sites for control of rates of accretion and resorption to be seen at a glance. For example, the deposition of extracellular collagen could be controlled by such varied factors as change in transmission of nuclear information, availability of energy, availability of substrate, variations in the biosynthesis of amino acids from glucose, or release of collagen from storage vacuoles. Similarly, resorption rate is clearly dependent on rates of acid production, biosynthesis of enzymes, and the mechanisms for delivery of enzymes into the extracellular space.

These thoughts, in turn, lead to a very simple classification for various bone diseases (table XIV). There are just three kinds of disturbances that can occur: (1) a defect in cellular machinery, (2) a deficiency in the availability of raw materials, or (3) a disturbance in the controls that regulate the rates of one or more steps; the latter disturbance can be subdivided into three categories: (a) hormonal, (b) local, and (c) "informational." This scheme with examples of diseases that, according to present information, fit into each category are shown in table XIV. Osteogenesis imperfecta, in which collagen synthesis seems to be deficient or deranged, appears to be a good example of a defect in cell machinery. Nutritional deficiency has been illustrated by osteomalacia, but scurvy could be cited equally well. The hormonal example is obvious, but perhaps the choices for the "local" and "informational" categories need some defense. Osteoporosis was picked for the former category because there are aberrations of the metabolic pattern in the bone cells in such patients, but so far no cause outside of the cells has been found for these changes. Another example which might be even better would be "fracture." It was omitted because it seemed a local rather than a systemic bone disease. Myeloma has been used because it is a widespread diffuse tumor that often produces bone disease that, if our recent evidence can be believed, may well result from a redirection of cellular biosynthetic activity.

TABLE XIV  
CLASSES OF BONE DISEASE

Type	Example
(1) Defects of cellular machinery.....	Osteogenesis imperfecta
(2) Deficiency of substrates.....	Osteomalacia
(3) Disturbance of controls:	
(a) Hormonal.....	Hyperparathyroidism
(b) Local.....	Osteoporosis of disuse
(c) "Informational".....	Multiple myeloma

RAISZ: Why not put myeloma under (2)?

NICHOLS: Because we have some experimental data which suggest that it should not be.

BUDY: What about genetic defects? Do you put them all under cellular machinery defects?

NICHOLS: Yes, I have, Dr. Budy, on the basis that most genetic defects eventually turn out to be enzyme deficiencies.

FREMONT-SMITH: That is information, is it not?

NICHOLS: I put it where I did because I have recently obtained some experimental information suggesting that genetic defects are probably not nutritional.

PRITCHARD: Where would an osteogenic sarcoma come into the system?

NICHOLS: I am not sure, but at present I would put it with myeloma.

PRITCHARD: What about infection with viruses and bacteria?

NICHOLS: I do not know. Where should I put them?

PRITCHARD: In nutrition?

URIST: Make a special category for infection.

FREMONT-SMITH: And have another one for miscellaneous.

NICHOLS: One must have a scheme in mind to guide one's work, and this is the scheme under which we have studied 103 patients with all sorts of diseases. In some instances, there are enough patients and the results are consistent enough for a pattern to be assigned to a disease; for example, hyperparathyroidism. Since we have already published these findings (ref. 152), I will not dwell on them. Instead, I would like to present some data on osteoporosis, in the hope that you will be able to help me understand what they mean; then I would like to go on to the skeletal response to fluoride. We will show an animal model, as well as some human data. Dr. Peck also has some information which I hope he will bring out (ref. 153) and thus move the discussion to the changes induced by multiple myeloma.

Listed in table XV are bone metabolic data for 17 patients with osteoporosis and who range in age from 39 to 78 years (ref. 154).

Three patients were treated with sodium fluoride for 1 year prior to biopsy, and one was studied before and after 1 year of sodium fluoride treatment. The metabolic data included in this table were derived from *in vitro* studies of biopsy samples and include measurements of several aspects of bone-cell function summarized in our hypothetical cell. The rate of collagen synthesis was measured in two ways: (1) from the incorporation of labeled proline, which reflects synthesis from preformed amino acids, and (2) from glucose, which measures amino acid as well as protein synthesis. Accumulation of label from these substrates in the cell fraction reflects a variety of cellular ac-

TABLE XV. — METABOLIC DATA FOR SOME OSTEOPOROSIS PATIENTS<sup>a</sup>

Patient	Sex	Age	DNA/100 mg wet weight	O <sub>2</sub> uptake, $\mu$ moles/ hr/mg DNA	Lactate, $\mu$ moles/ hr-mg DNA	Proline, $\mu$ moles/hr/mg DNA		Glucose, $\mu$ moles/hr/mg DNA	
						Cells	Collagen	Cells	Collagen
H-21.....	F	64	0.198	0.48	0.59	30.7	0.60		
H-24.....	F	48	.198	1.05	1.09	22.9	1.29		
H-29.....	F	78	.107	.65	1.40	12.6	.87		
H-30a.....	F	57	.137	.72	1.16	39.0	.47		
H-30b.....	F	58	.054	.78	.74	57.6	.28	1321.2	5.14
H-32.....	F	67	.241	.00	.92	53.2	.06	556.5	.43
H-34.....	M	68	.167	.56	.74	22.6	.13		
H-35.....	F	47	.161	.91	1.39	46.3	.33	192.6	5.23
H-38.....	M	61	.140	.71	.80	26.8	.08	156.6	3.19
H-40.....	M	42	.187	.98	.64	11.4	.13	179.8	2.90
H-42.....	M	39	.121	.00	.40	56.5	.12	392.1	1.27
H-44.....	F	65	.126	.00	.80	15.2	.13	262.9	4.92
H-46.....	F	64	.162	.10	.81	101.0	.17	1167.0	5.49
H-48.....	F	71	.084	.00	.90	19.3	.16	182.9	2.56
H-49.....	F	53	.150	.54	.92	38.7	.16	311.6	5.53
H-50.....	F	44	.187	.41	.72	44.3	.12	950.9	7.66
H-53.....	M	57	.108	.50	.89	50.6	.25	445.6	6.44
H-71.....	M	62	.104	1.05	.85	33.0	.21	442.8	16.10
Normals: <sup>b</sup>									
Mean.....			.188	.47	.79	23.4	.24	320.0	4.00
S.D.....			.290	.22	.29	7.84	.09	170.0	.92
Number of samples.....			6	8	6	5	5	4	5

<sup>a</sup> It should be noted that errors in the values for the standard deviations of the values in normals presented in the original publication (ref. 154) have been corrected here.

<sup>b</sup> Age of normal subjects: 17-43 years; sex of normal subjects: 5 males, 3 females.

tivities. We now know (ref. 155) that proline label is to be found concentrated in the cell sap as the free amino acid, as well as incorporated into collagen and constituent cellular proteins. While some proline label is probably present in metabolic intermediates as well, much more of the glucose label is to be found in such materials, as Cohn and Forscher (ref. 156) first showed. We also know that glucose label must be present in free amino acid (ref. 157), in nascent collagen, and in cell proteins as well. Thus, glucose label in cells reflects many functions and probably should be taken as an index of overall metabolic activity. Finally, energy metabolism is estimated in two ways: (1) by  $O_2$  uptake and (2) by lactate production, the latter perhaps being related to bone-mineral resorption as well.

At the bottom of the table, the means of values derived from eight normal subjects together with their standard deviations are shown for comparison. While bone from eight normal subjects was studied, data in some categories were collected from as few as four. The combination of the small number of subjects and of the considerable variability in metabolic activity that seems to occur from sample to sample resulted in standard deviations as large as 50 percent of the mean. Yet despite this, at least one and sometimes several of the metabolic variables measured was more than two standard deviations away from the normal mean in all but 3 of the 17 patients studied. Table XVI will make this clearer.

BAUER: How about the normals? Were they normal in all respects? They need not be, you know.

NICHOLS: That is perfectly true. Data for eight normals are shown. Since this table was prepared, we have done three additional normal subjects.

If they are included in the means they are very similar, as are the standard deviations. The latter are a bit smaller, but not much. The total normal group now ranges in age from 17 to 43 years and includes five men and three women. All were patients hospitalized for surgery which involved the skeleton, but who did not have any evidence of systemic bone disease. Nor were they people with recent fractures; this is important, as we have since discovered.

FREMONT-SMITH: Why is this important?

NICHOLS: That they did not have fractures is important because the presence of a recent fracture, even at some site remote from the biopsy area, seems to stimulate bone metabolic activity.

BAUER: It seemed, from table XV, that every osteoporotic individual studied was abnormal, i.e., outside the normal range, in every one of the variables studied. How abnormal were the nonosteoporotic subjects?

NICHOLS: This is a compilation of all the normal data available

at the time. The gaps occurred because of broken tubes, insufficient bone to measure everything in a given individual, and so forth.

HEANEY: In which way were they abnormal? Were they just outside the standard deviation in both directions?

NICHOLS: Yes, and this is what we are going to discuss in table XVI.

MACDONALD: Are these biopsies?

NICHOLS: Yes; the majority were taken from the iliac crest.

HOWELL: How many grams of bone does it take for that battery of studies?

TABLE XVI  
OXYGEN CONSUMPTION

Sample no.	Lactate	Cells		Collagen	
		Glucose	Proline	Glucose	Proline
		Low			
32.....	n	n	+	—	+
42.....	n	n	+	—	n
44.....	n	n	n	n	n
48.....	n	n	n	n	n
		Normal			
21.....	n	—	n	—	+
29.....	+	—	n	—	+
30a.....	n	—	+	—	+
30b.....	n	+	+	n	n
34.....	n	—	n	—	n
38.....	n	n	n	n	n
46.....	n	+	+	n	n
49.....	n	n	n	n	n
50.....	n	+	+	n	n
53.....	n	+	+	n	n
		High			
24.....	n	—	n	—	+
35.....	+	n	+	n	n
40.....	n	n	n	n	n
71.....	n	n	n	n	n



NICHOLS: When we started these studies we needed about 1½ grams. Recently, we have refined our methods so that we can do most measurements with 300 milligrams. If collagenase activity is also measured, we need about 500 milligrams. There are some additional problems that arise when the sample size is very small. For example, the total cellularity of this tissue is quite low, so there is a risk of not having a representative sample of the several types of cells; the total number of cells is too small. At least this seems the best explanation for the increasing variation one gets as the sample size decreases.

Faced with the kind of scattered data shown in table XV, the next step is to see whether clear patterns emerge when the patients are classified on the basis of one or another metabolic variable. The next three tables illustrate such possible classifications.

Table XVI shows what happens when patients are grouped according to bone-cell oxygen consumption. Three groups can be distinguished with low, normal, or high values depending on whether their values fall within, below, or above two standard deviations on each side of the mean value found in samples from normal subjects. Although the majority fall within the normal range, four subjects with low rates of O<sub>2</sub> uptake and four subjects with increased rates can be identified. However when the results of other metabolic studies are examined, no very consistent pattern is found. About all that can be said is that when O<sub>2</sub> uptake is low, collagen biosynthesis is apt to be normal or low, while the label retained in cells is generally normal or (as in two cases) increased. In patients with normal bone-cell O<sub>2</sub> uptake, other data also tend to fall in the normal range. What changes are present tend to be above the normal range; the same is true when O<sub>2</sub> uptake is abnormally high.

Clearly, this classification does not bring out any very well defined patterns of change. Similarly, classification based on rates of collagen biosynthesis from glucose (table XVII) or proline (table XVIII) fails to identify any clear-cut pattern of disturbed metabolism. Again, most of the patients fall into the normal category of each classification.

When other classifications are tried, the results are equally disappointing. Indeed, the only common denominators which appear are that cellular-label retention from both glucose and proline is either normal or high, even in people with normal or low rates of collagen synthesis from the same precursor.

Incidentally, several of these patients had received sodium fluoride treatment for upward of a year at the time of biopsy. One patient was studied both before and after 1 year of treatment with 100 milligrams sodium fluoride by mouth each day. It was interesting that the metabolic patterns in these patients could not be distinguished from the

TABLE XVII  
COLLAGEN SYNTHESIS FROM GLUCOSE

Sample no.	O <sub>2</sub> uptake	Lactate	Cells		Collagen from proline
			Glucose	Proline	
	Low				
32.....	—	n	n	+	—
42.....	—	n	n	+	n
	Normal				
30b.....	n	n	+	+	n
35.....	+	+	n	+	n
38.....	n	n	n	n	n
40.....	+	n	n	n	n
44.....	—	n	n	n	n
46.....	n	n	+	+	n
48.....	—	n	n	n	n
49.....	n	n	n	n	n
	High				
50.....	n	n	+	+	n
53.....	n	n	n	+	n
71.....	+	n	n	n	n

rest. Some changes did occur, however, in patient 30 (see table XV) during her year of treatment, a period over which she developed roentgenographic evidence of fluorosis of the skeleton.

The question, of course, is "What do these data mean?" My answer at present is, unfortunately, "I do not know," but perhaps you will be able to help me understand them.

URIST: May we call for some questions now about what you have covered so far?

NICHOLS: I was going to ask Dr. Heaney or Dr. Bauer to tell me how they would attack the problem from here. I find myself at a loss.

HEANEY: Before you call for questions, I would say your statistics are unmanageable. There is nothing in your presentation that could not be accounted for by random chance; this is not to say that real

TABLE XVIII  
COLLAGEN SYNTHESIS FROM PROLINE

Sample no.	O <sub>2</sub> uptake	Lactate	Cells		Collagen from glucose	
			Glucose	Proline		
32.....  30b..... 34..... 35..... 38..... 40..... 42..... 44..... 46..... 48..... 49..... 50..... 53..... 71.....	Low					
	—	n	n	+	—	
	Normal					
	n	n	+	+	n	
	n	n	—	n	—	
	+	+	n	+	n	
	n	n	n	n	n	
	+	n	n	n	n	
	—	n	n	+	—	
	—	n	n	n	n	
	n	n	+	+	n	
	—	n	n	n	n	
	n	n	n	n	n	
	n	n	+	+	n	
	n	n	n	+	n	
	+	n	n	n	n	
	High					
	21.....	n	n	—	n	—
	24.....	+	n	—	n	—
	29.....	n	+	—	n	—
30a.....	n	n	—	+	—	

forces were not involved. I am merely talking about interpreting the data.

NICHOLS: I have also come to this conclusion.

RAISZ: I would like to ask about the problem of age. Your normals ranged from age 17 to 43 years, and I think the patients were all older except for one patient of 41. Was this a lady who had had her gonads removed?

NICHOLS: Surprisingly enough, it was a man.

RAISZ: The question remains as to whether there is variability with age in these measurements.

NICHOLS: We cannot entirely determine this. We suspect that very young people have a much faster bone metabolic rate; but we have relatively few data from children, and most of these are abnormal children, because it is very difficult to get a normal child's bone in adequate quantity. The few samples that we have studied show variable higher rates, sometimes as much as threefold or fourfold the adult normal as defined by these numbers.

HOWELL: You refer to DNA as a standard of reference?

NICHOLS: Yes; DNA.

FREMONT-SMITH: What with automobile accidents and all, I should think you would have a lot of normal bone available, even from children.

NICHOLS: The problem is that people are rarely autopsied until some hours after death, and by this time the tissue has deteriorated, unfortunately.

FREMONT-SMITH: But people are operated on; amputations and all kinds of things are happening. There would seem to be a real opportunity if one could alert a group of surgeons to the kinds of specimens you want and to what these specimens should be put into.

HEANEY: I think, Dr. Fremont-Smith, it would be very important to work entirely with bone from exactly the same region. We have enough variability without taking a hit-and-miss sample from a leg here, a finger here, and a finger there.

FREMONT-SMITH: There are quite a lot of legs, and I suspect one would have not four to seven normals but perhaps 40 or 50 normals in a few years. One of the situations evident here is that the sample of normals is completely inadequate to base anything on. I am sure a careful effort with accident cases coming into operation would provide at least a better sample of normals than Dr. Nichols has.

NICHOLS: Obtaining good normal materials is indeed the most critical issue at this time. Dr. Peck, did you have a question?

PECK: I would like to ask about the effect of anesthesia on the metabolism of surviving cells.

NICHOLS: I do not know.

PECK: Do you have any patients that were operated on under various types of anesthesia?

NICHOLS: The biopsies were obtained under a variety of anesthetics, most of them general anesthesia. I have not examined the data in relation to the anesthetic agent; it should be done.

BAUER: What, by definition, do you mean by "osteoporosis"? For those who do not work in this field, may I explain that there are several current definitions of osteoporosis; whatever definition one chooses, it is

still difficult to distinguish between osteoporosis and nonosteoporosis.

NICHOLS: This group was defined as follows; they had pain.

BAUER: Where?

NICHOLS: The patients had crushed vertebrae, as shown by roentgenographs. They had normal serum calcium and phosphorus.

BAUER: My other question is "Have you done this kind of analysis on bone biopsies in experimental animals?"

NICHOLS: We have data from rats, pigs, guinea pigs, and rabbits, but none from dogs or cows.

BAUER: Even though you do not have too much normal material (this is the same practically for whatever one tries to do in studies of disease—it is difficult to get normal materials), I am sure that the biochemical methods, or other methods, used are much more difficult to handle than the logistic problem of getting normals. When you compare the changes that you find in your few normals with those of the many nonnormals, are these comparisons in approximately the same range as those in your animal experiments?

NICHOLS: The normal range appears, so far, to be wider in man than in animals, but I think this probably is a function largely of number of samples examined.

ROBINSON: In the investigations of bone that you obtain at the time of surgery, there are apt to be, particularly in the osteoporotic individual, many other cell types in the whole bone specimen besides osteoblasts, osteoclasts, and osteocytes. Do you have any way of standardizing your sample so that you know how much actual bone tissue is in this specimen of whole bone? It appears that you have a DNA ratio per gram of wet weight, and a lot of the DNA might be associated with cells that are not concerned directly with bone accretion, resorption, or maintenance.

NICHOLS: This is basically the problem of marrow contamination, which is with us in all our work with animals as well as man. The answer to your question is not entirely adequate, but perhaps if I explain the details of our procedures you will see how we try to minimize the contamination with marrow and other nonbone cells.

The biopsy, when taken, is chilled immediately in Krebs-Ringer buffer at 2° to 3° C and transported at that temperature in buffer to the laboratory; there it is carefully scraped free of soft tissue. Iliac crest samples, at least, are a mixture of a thick plate of cortical bone with trabecular bone attached. Both are finely minced with a bone rongeur and then shaken quite vigorously with cold buffer. By the way, Dr. Talmage tells me that this is the way he gets osteoclasts out.

TALMAGE: Roughly.

NICHOLS: We use this technique to wash out cells that look like marrow on smear. If washed three times, a piece of bone which

was red is now pale pink or white and on histologic examination has lost most of its marrow elements.

ROBINSON: And then you weigh that material?

NICHOLS: Right. Then after incubation, the cell fraction is separated chemically.

BAUER: Have you had an opportunity to compare your data obtained on biopsy material with data obtained on autopsy material; i.e., from individuals who have been dead for 24 hours?

NICHOLS: Actually, we have done only two post mortem samples, both traumatic. Neither showed much, if any, activity, as I recall. However, this was some time ago, and perhaps we should go back and try again.

Paget's disease is something we have yet to study. We have looked at the influence of fractures once, in a patient with osteogenesis imperfecta. We have also examined isolated cases of other diseases. I showed you osteoporosis partly to see if somebody could help me find an interpretation of the data and partly because our findings indicate a completely polyglot group of cellular disturbances; this is important because osteoporosis is a polyglot disease.

RAISZ: I do not believe that the complexity of your data rules out a simplistic theory of osteoporosis. Although this theory may not be correct, I would like to hear the reaction of this group to the possibility that there is a single mechanism for the development of osteoporosis. I think that the variations which you have observed could occur on the basis of the heterogeneity of the population of cells that we have been looking at.

MACDONALD: Do those data not show that there is a need for a method that would establish whether the osteopenia is progressing or whether it is quiescent? Perhaps this distinction in these patients would simplify some of the comparisons that may bother you. Methods of determining whether the osteoporosis is active or quiescent might open a new area for discussion.

PECK: I think it would be very valuable, if feasible to obtain; the reproducibility of the observation in the same patient from day to day, week to week, and even month to month.

NICHOLS: This is something we have not done. Patients are reluctant to be biopsied a second time; so far I have not asked a patient for a third biopsy. The amount of bone needed is still too great and the procedure too traumatic to justify such repetitive studies at present.

HEANEY: You are to be congratulated for attempting this exceedingly difficult task; I think it has to be done. Also, I think it is this sort of approach that is going to provide us with valuable information, but I am convinced from what I have seen so far that much of the discussion of the last 2 or 3 minutes is completely irrelevant. We do not know

whether the data are themselves dispersed because we have no adequate standard of reference. I do not see how we can conclude anything except that this is a valuable first step, and we hope we can go on from here.

I think osteoporosis is a diverse disease with many mechanisms, many pathogeneses, and many manifestations, but I do not think your data show it one way or the other.

NICHOLS: I agree with you absolutely. We do not have an adequate standard of reference yet. Moreover, we do not know enough about the details of the cellular mechanisms to begin to interpret what we see.

PRITCHARD: Would you like to know the ratio of osteoblast to osteoclast activity? Suppose you took the ratio of alkaline phosphatase to acid phosphatase activity as a measure of the ratio of osteoblasts to osteoclasts. Surely, that ought to be a useful index.

URIST: In the serum or the supernatant?

PRITCHARD: In the bit of bone.

NICHOLS: We have made some measurements in other systems, but not in this particular one.

PRITCHARD: You could take the ratio and call it the Pritchard Index.

TALMAGE: The osteocyte also has these enzymes, so why should you be considering only the osteoblasts and osteoclasts?

PRITCHARD: If the index is a useful guide as to whether the bony condition is progressive or not, let us not worry too much about the osteocytes.

FREMONT-SMITH: Have you had any samples of bone from disuse, for instance, from polio cases?

NICHOLS: No; we have not. There is so little paralytic polio now that we rarely have such patients, and it is very hard to come by an adequate sample.

I would like to turn the discussion now to resorption. So far, we can only say that these patients have osteopenia by roentgenographic examination. The only thing that tells us that resorption might be increased is that one cannot make a case for anything else happening on a regular basis. However, now we have a few measurements of collagenase activity in human bone and this permits us to categorize one small group of osteoporotic individuals more precisely in terms of resorptive activity.

The essence of the technique is similar to that we have described for animal bone (ref. 158). One takes human bone, grinds cells out of it, homogenizes the cells with Triton-X-100, which breaks up the lipid membranes, and incubates this mixture with  $^{14}\text{C}$ -labeled bone collagen for 1 hour. Then the mixture is diluted with ice-cold water

and poured into an ultrafilter. The ultrafiltrate from the incubation mixture is then dried, taken up in a small volume, and counted.

It turns out that the counts in the ultrafiltrate are in proline and hydroxyproline, when the collagen has been labeled with  $^{14}\text{C}$ -proline. These counts are present in the same proportions as they are in collagen, and their specific activities are similar to what they were in the parent molecule, so that it seems likely we are measuring collagenolytic activity. The amount of such activity seems to vary directly with the DNA content of the homogenate in normals. The mean value obtained in five such individuals is 8.75 milligrams collagen broken down per milligram DNA, with a range of  $\pm 2$  S.D. on each side of the mean of 4.4.

At present we have two measurements in patients with osteoporosis; one was 62 years old and the other was 57. The latter is from a lady who had been treated with estrogen for some time and had typical postmenopausal osteoporosis. The other one is from a man with coronary arteriosclerosis and marked osteoporosis—possibly some form of osteogenesis imperfecta, because he had a crushed vertebra when he was only 33. The point is that these data are just enough to suggest that an elevated bone collagenase activity may turn out to be still another way of classifying osteoporotic patients.

TALMAGE: Could I ask one thing concerning your data? Were the samples from the osteoporotic individuals taken from the same part of the same bone as the samples from the control patients?

NICHOLS: Yes.

TALMAGE: All of these were from iliac crests?

NICHOLS: Almost all. There is one other thing that ought to be said; the metabolic activity per wet weight unit, as generally defined, varies greatly depending on where one takes the bone sample—head of the femur, iliac crest, or rib. However, on the DNA basis the metabolic variables do not vary much.

URIST: DNA is a valid index of cellular activity.

ARNAUD: You really do not know if some of the results obtained might actually be the result of fewer cells doing more work.

URIST: Whatever the interpretation, Dr. Nichols is quantitating the cellular activity in the sample. The method should appeal to Dr. Bauer because he has changed the name of the disorder from osteoporosis to osteopenia.

BAUER: If you have two patients both suffering from osteoporosis and one patient is a 36-year-old man and the other a 70-year-old woman, you must be very cautious in concluding that these two individuals are suffering from one and the same disease. If we define osteoporosis as a condition of too little bone and fractures, then many different



mechanisms may produce this condition. Etiologically then, the 36-year-old man does not suffer from the same condition as the 70-year-old woman.

ARNAUD: I do not think that follows at all. I think they may be the same.

BAUER: Dr. Nichols, I am most intrigued by attempts to miniaturize your bone-biopsy analyses. Do you see what I am driving at?

NICHOLS: I think so. One can just go on making measurements because one knows how, but I am not sure this is a terribly valuable approach to the problem. This is the reason that I am beginning to think that maybe the thing to do is to leave shrinking elderly ladies alone until we know a bit more about normal bone-cell processes.

URIST: Perhaps I can arouse some discussion from Dr. Currey, Dr. Sjöstrand, Dr. Bélanger, and some of the others who do not see patients with osteoporosis every day, by showing a few figures to illustrate the nature of the problem.

BUDY: For the record, would someone please define "osteopenia"? A working definition would be most useful at this time.

BAUER: Too little bone.

FREMONT-SMITH: Good.

CURREY: The sturgeon has osteopenia.

HEANEY: As long as someone asked this question, I think when we say "too little" we are referring to some frame of reference. Too little for what?

BAUER: For the normal.

ROBINSON: The definition ought to be "too little bone tissue for a unit volume of whole bone."

HEANEY: For what?

BAUER: I think the simplest is "too little bone, as compared with normal." It does not disturb me too much that this cannot be measured very accurately at present.

HEANEY: It does not disturb me that it cannot be measured out, but when we say "too little" it means deficiency somehow, and the very word "deficiency" means some frame of reference. Is it too little for structural purposes?

MACDONALD: Yes.

HEANEY: I quite agree. Is it too little for homeostatic purposes?

URIST: Besides being too little in amount, the bone in the aged patient with typical osteoporosis is defective, degenerate, in part, dead tissue. I will substantiate these statements with some figures of patients I have seen in the clinic.

Figure 86 shows a 67-year-old woman with a typical form of severe senile osteoporosis in the lateral view (left) and the posterior view (right). Note the increase in the length of arms relative to the length

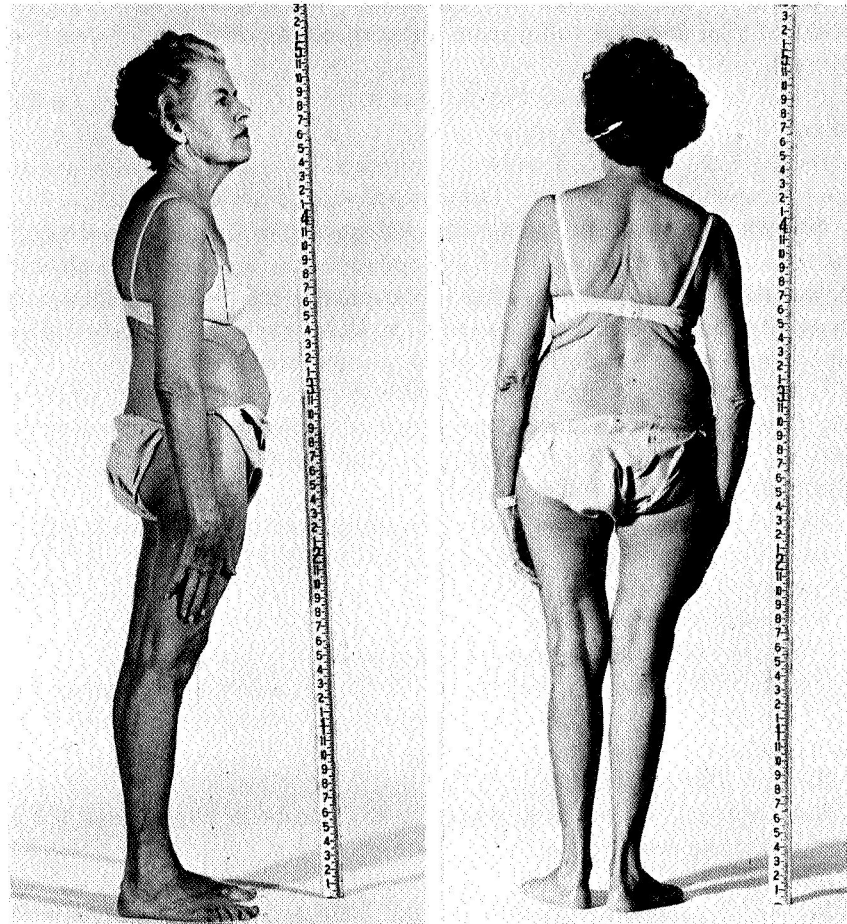


FIGURE 86. Photographs of a 67-year-old woman with a typical form of severe senile osteoporosis.

of the torso. Also, note the deep circumferential skin folds just below the costal margins. When the patient was measured with a yardstick at monthly intervals, it was clear that her height decreased progressively, and over a period of 2 years she lost approximately 3 inches in the length of the torso.

In view of the location of the gross changes in the spinal column, the best place to do a biopsy on a patient with osteoporosis would be on a vertebral body. Unfortunately, this is not readily accessible or suitable for obtaining an ample specimen. The next best place is the proximal end of the femur on the lateral aspect or the medial aspect of the upper tibia. It is customary to assume, but by no means proved, that the disease is qualitatively the same in all parts of the skeleton.

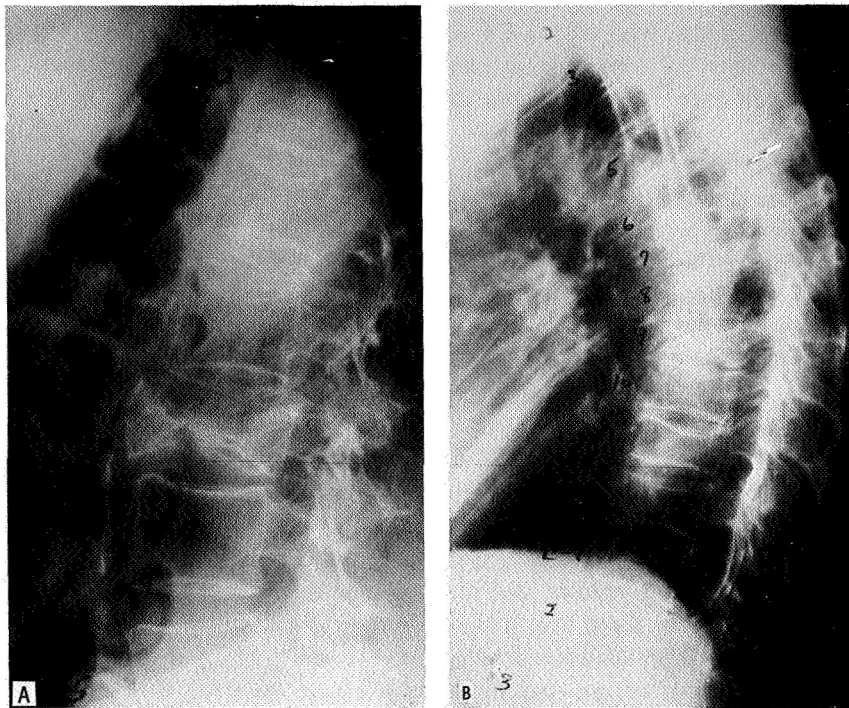


FIGURE 87. Roentgenographs of osteoporotic spines: (a) Lumbar spine and (b) dorsal spine.

Figure 87(a) is a roentgenograph of an osteoporotic lumbar spine showing ballooned disks, thin anterior cortex, collapse of the third lumbar vertebra, and balloon-shaped intervertebral disk spaces. Figure 87(b) is a roentgenograph of the dorsal spine of a typical patient with senile osteoporosis. Note the collapse of the 6th, 7th, 9th, and 11th thoracic vertebrae and the upper dorsal kyphosis.

A reduction in trabecular bone mass, as well as cortical bone, occurs and can be determined by measuring the density of the vertebral body as an organ (apparent vertebral density).

The procedure on autopsy specimens, as employed by Dr. Michael Gurvey, a postdoctoral fellow in the Bone Research Laboratory at UCLA, is as follows: (1) the vertebral bodies are scraped clean of ligaments and weighed on an analytic balance, and (2) the volume of the vertebral bodies is determined in water, by use of the volume of water displaced by the bone, expressed in cubic centimeters. The specimens are then prepared and analyzed for apparent density as follows:

1. The core of the center of the vertebral body consisting of only spongiosa is removed with a 2.6-centimeter hole saw.

2. The core of spongiosa is washed under a stream of cold water to remove all soft tissue elements (bone marrow, vessels, blood, and so forth); it is then cut into disks of 1 to 2 centimeters in thickness with a band saw.

3. The disks of spongiosa are agitated for 24 hours in cold tap water on a shaking machine to remove soft tissue cells. When the process is complete, the color changes from yellow to white.

4. The disks of washed bone are defatted in a Soxhlet apparatus by refluxing for 24 hours in a 2-to-1 solution of chloroform and methanol; then they are dried in an oven at approximately 80° C for at least 72 hours or until they reach a constant weight.

5. After weighing on an analytic balance, the volume of each disk is determined by measuring the dimensions with a sliding caliper and using the formula,  $r^2$  times height; the volume is expressed in cubic centimeters.

6. Apparent density of the spongiosa disks is then calculated from the weight per unit volume and in grams per cubic centimeters.

Five gradations from five different subjects are shown in figure 88. Only the subjects with bone density of 0.128 and 0.107 had spontaneous fractures, or pathologic osteoporosis. These patients had approximately 40 percent of the bone mass, or apparent density, of the average nonosteoporotic young individual at age 30.

Using the method described in the preceding paragraphs, the vertebrae were used to construct a spondylometer for use in patients with osteoporosis. A spondylometer is defined as a scale for comparing normal and abnormal radiopacity with the aid of the naked eye; it reveals the distribution, quality or structure, and quantity of bone tissue. Figure 89(a) is a roentgenograph showing the spondylometer and the lumbar spine of a 50-year-old nonosteoporotic woman. The radiopacity of the second lumbar vertebra corresponds approximately to the bottom vertebra, or to the most dense on the column of the spondylometer. The vertebra in the top of the spondylometer is from an osteoporotic patient and shows deformity from an old fracture. Figure 89(b) is a roentgenograph of the spondylometer and the lumbar spine of a 70-year-old woman with severe osteoporosis. The radiopacity of the first and second lumbar vertebrae correspond to the radiopacity of the top two segments of the spondylometer that were removed from autopsy subject with severe osteoporosis. The first lumbar vertebra has collapsed, while the second is biconcave.

We have also performed biopsy studies on patients with severe osteoporosis before treatment to exclude other disorders, such as osteo-

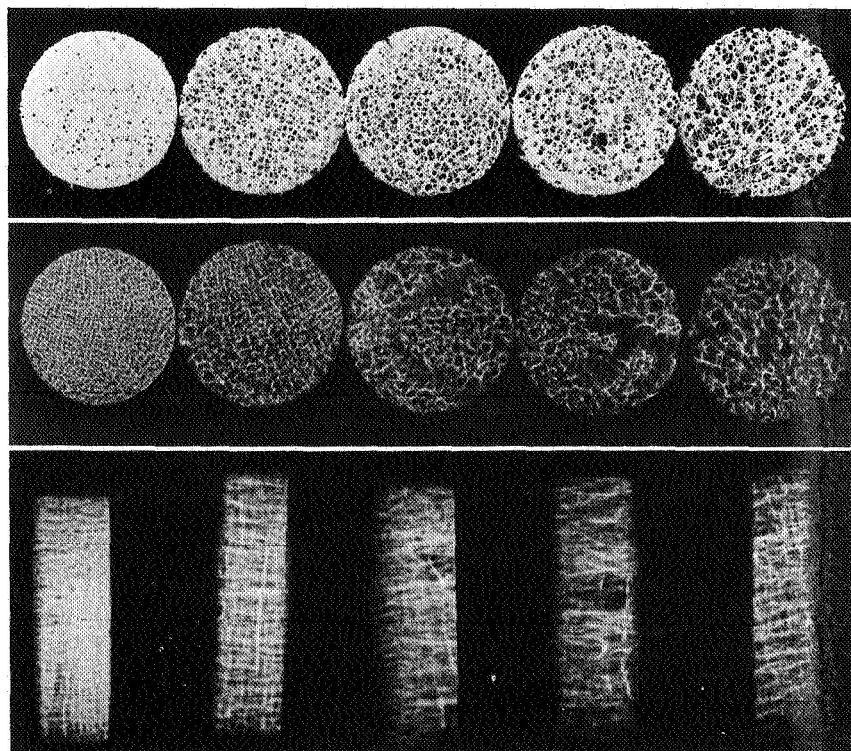


FIGURE 88. Photographs (top row) and roentgenographs (center and bottom rows) of specimens of bone from osteoporotic subjects taken from center of vertebral bodies with apparent densities (left to right) of 0.256, 0.243, 0.157, 0.128, and 0.107. Note that the trabeculae are not simply reduced in number. The vertical trabeculae increase in thickness as the horizontal trabeculae are resorbed. These changes in the structure of the spongiosa occur while the cortical bone decreases progressively in thickness.

malacia, osteitis fibrosa, multiple myeloma, and so forth (ref. 105).

FREMONT-SMITH: What bone did you biopsy?

URIST: The lateral aspect of the upper end of the femur; also the medial cortex of the proximal end of the tibia in patients with severe osteoporosis of the spinal column. Unfortunately, the spinal column is inaccessible for biopsy on aged individuals. We made cell counts, which are summarized in table XIX and observed an overall difference of 6 percent more dead bone in the tibia and femur of the osteoporotic than the nonosteoporotic. The difference may be greater in the vertebral bodies of the two groups, but we do not have data to prove this.

NICHOLS: This is dead bone as judged by empty lacunae or calcified cells?

URIST: Yes.

HEANEY: Dr. Nichols, why are you reluctant to call that dead bone?



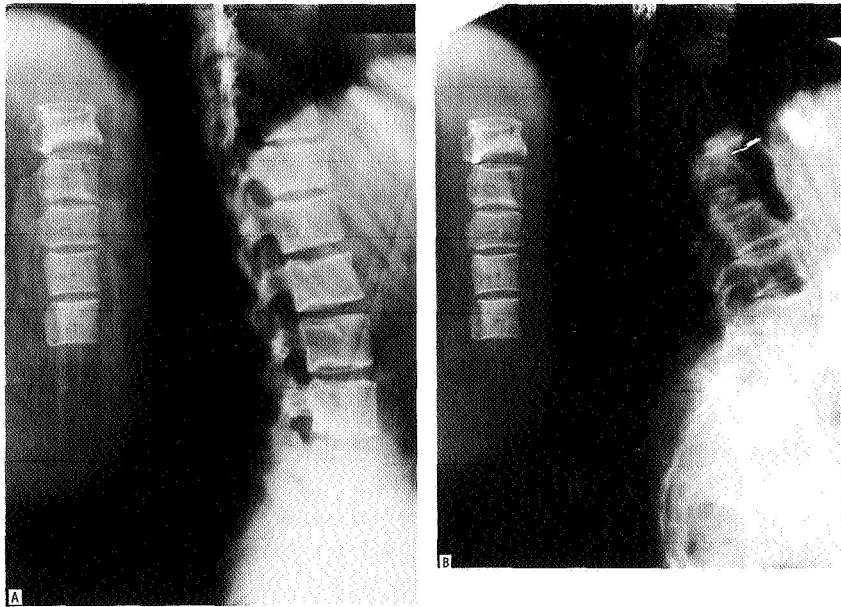


FIGURE 89. Roentgenographs of the spondylometer and lumbar spine of (a) 50-year-old nonosteoporotic woman and (b) a 70-year-old woman with severe osteoporosis.

NICHOLS: Because I think that just because a particular individual cell or set of cells is dead, it does not mean that the bone is necessarily dead any more than that it is necessarily alive when it has an intact cell in it. This is important because what we are talking about right now is a cell type that has a job to do which it is or is not doing. Cells on the surfaces may be performing quite normally while some in the depths may not, and vice versa. Really, all bone can be called "dead" because most of it is nothing but a collection of extracellular collagen which is calcified.

CURREY: Oh, no, no.

FREMONT-SMITH: The keywords were "nothing but." If you had left out the "nothing but," everybody would agree.

CURREY: By what criterion do you say there is no difference between a bone that is chock-full of healthy osteocytes and bone that is chock-full of mineralized osteocytes? Are these the same as regards life and death?

NICHOLS: Clearly, not so far as the osteocyte is concerned. I say calling a bit of bone alive or dead so far as the calcified matrix is concerned is an improper use of the term.

CURREY: But you are only talking about 99 percent of bone when you are talking about calcified matrix, and the other percent is very important.

NICHOLS: Agreed. What I am saying is that we should refer to an osteocyte as dead, rather than saying the bone is dead. Do you agree with this thought?

BÉLANGER: Oh, of course, very much so, because this sort of thing can be followed by a lot of activity at the surface, either bone growth or osteoclasia, and these two types of cells in the bone are not dead at all. The osteocytes are dead inside.

NICHOLS: I think this has great implications in how tissue behaves.

HEANEY: But that microscopic volume is certainly dead. One does not say the whole femur is dead because it has a few microscopic areas of empty lacunae.

HOWELL: Is the method of fixation and embedding such that you are not knocking a soft little particle out of a solid sheet of bone? There could be some difference in ease of removal of the soft part rather than actually not being there in the first place.

URIST: Fixation and artifacts are certainly a cause for concern. However, the empty lacunae in an osteoporotic individual are also actually enlarged. This is described in the old literature in various bone diseases as slow necrosis, oncosis, cell necrobiosis, or osteonecrobiosis. There are all gradations of oncosis. It occurs in normal aging of bone and accounts for a steady increase in the amount of dead bone in the skeleton with time. The question is whether the process is accelerated, accentuated, or exacerbated in osteoporotic compared with nonosteoporotic persons.

HOWELL: If your thesis is correct, that the axial skeleton lumbar spine is the seat of the worst disease, autopsy changes should not alter this hard-tissue phenomenon you describe, and therefore you ought to be able to document how consistent or how prevalent these changes are in the axial skeleton.

CURREY: There were two figures you mentioned, of 28 and 22 percent dead cells, which you said was not a great difference, but the two types were very consistent. This surprises me. I do not know what your standard deviations were, but for the counts that I did on healthy nonosteoporotic people, I would certainly never have been able to distinguish between 28 and 22 percent, except perhaps on an enormous series; however, you are quite happy that the variance was sufficiently small for you to distinguish between them.

URIST: The 6-percent difference is based on a sample of 10 000 cells counted by three people working independently.

CURREY: This is just in two different people?

URIST: We have biopsies on 24 cases of severe osteoporosis and on 10 cases of nonosteoporotic persons of comparable age with osteoarthritis in various joints.



PRITCHARD: This material is very difficult to fix adequately; I thought that some of the spaces you said were empty had cells in them, but there seemed to be some that showed fixation artifacts.

URIST: You are quite right; but to deal with this possibility the retracting osteocytes were counted separately, as shown in table XIX.

PRITCHARD: Were they reactive? There is the possibility of error here.

URIST: Let us go on and see more material from autopsy subjects.

BÉLANGER: May I say something on this topic of empty lacunae and dead osteocytes? This is a phenomenon which one can obtain progressively with hyperparathyroidism in animals, such as we have done with Dr. Krook at Cornell on horses. As the disease or syndrome progresses, the number of these things increases, and one can follow also the death throes, if I may say so, of the osteocytes in this type of material, so it is certainly no artifact.

URIST: McLean and Bloom (ref. 140) described osteonecrobiosis in experimental hyperparathyroidism in rats.

A good survey of pathologic osteoporosis on autopsy subjects is difficult to complete. The reason is that patients with severe pathologic osteoporosis are long-lived and healthy; therefore, they appear relatively infrequently in university teaching hospitals for autopsy. We would like to obtain data like that shown in figure 90 from 50 patients with severe osteoporosis, but we find that less than 1 percent of autopsy subjects have severe osteoporosis comparable with that found in 85-year-old white healthy females.

NICHOLS: How did you get the marrow out? Are the specimens dried first?

URIST: The marrow was removed by cold-tap-water agitation on a shaking machine, and then refluxed for 24 hours in chloroform and methanol in a Soxhlet apparatus.

PECK: Do you have any technique for measuring the interstitial volume? I should not have said "interstitial volume," I should—

URIST: We have not measured the intertrabecular interstitial volume, but that could be done.

NICHOLS: Your point is important because in our data we cannot find any difference in metabolic activity on a DNA basis between the ages of 40 and 80. If anything, the cells are a little more active in older people.

URIST: The cells may be more reactive, but in terms of the net change, bone mass diminishes with age. Reduction of bone mass with age has been measured by Trotter et al. (ref. 159) on whole bones, Arnold (ref. 160) on blocks of bone, and Jowsey (ref. 161) on microradiographs.

I think there is some—but not much—stable information in this

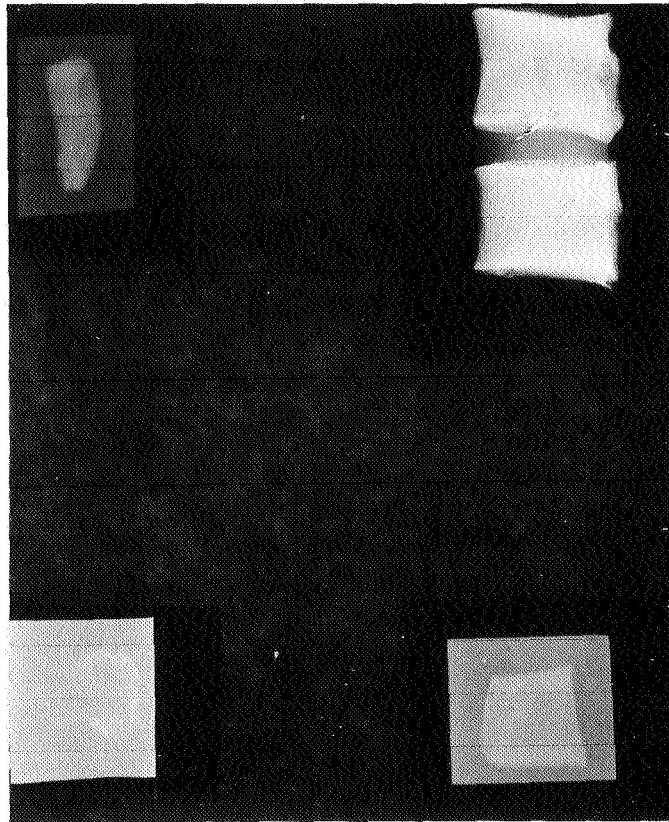


FIGURE 90. Radiographs showing a four-organ preparation from a nonosteoporotic autopsy subject, male, age 67. The tissue in the upper left is skin; lower left, fascia; upper right, first and second lumbar vertebrae; lower right, aorta. Samples of tissue from the four organs were analyzed for total calcium, total phosphorus, hexosamine, and collagen in an effort to determine whether mineral was transferred from bone to soft tissue in patients with osteoporosis. The results on approximately 39 consecutive autopsy subjects were equivocal.

field that is valuable and that we can respect. I do not think everything is chaos.

Tables XX and XXI demonstrate that approximately one of every four white healthy females, average age 85, in the United States, has severe osteoporosis; one of every seven debilitated, inactive, chronically ill white male, average age 66, has a moderate degree of osteoporosis. Statistics for healthy men in the seventh decade are not available. The period of life expectancy of the average male is less

TABLE XX  
CONDITION OF THE SPINE IN 100 CONSECUTIVE WOMEN (AVERAGE AGE 85)

Radiologic observations	Percent
Generally negative, good bone density.....	14
Spondylosis, good or increased bone density.....	60
Osteoporosis (diagnosis based upon collapsed vertebra).....	26
Compression fractures of dorsal spine.....	26
Biconcave lumbar vertebra.....	24
Compression fractures of lumbar vertebra.....	20
Compression fractures in both dorsal and lumbar spine.....	18
Fractures of the hip.....	15
Spondylosis and osteoporosis.....	12
Severe kyphosis with spondylosis.....	4
Severe kyphosis, osteoporotic (from fractures).....	14
Severe kyphosis, Scheuermann's type.....	1

TABLE XXI  
CONDITION OF THE SPINE IN 100 CONSECUTIVE MEN (AVERAGE AGE 66)

Radiologic observations	Percent
Generally negative, good bone density.....	9
Spondylosis, good or increased bone density.....	70
Osteoporosis (diagnosis based upon collapsed vertebra).....	18
Compression fractures of the dorsal spine.....	27
Biconcave lumbar vertebra.....	14
Compression fractures of the lumbar spine.....	15
Compression fractures of both dorsal and lumbar spine.....	9
Spondylosis (minimal) and osteoporosis.....	10
Severe kyphosis with spondylosis.....	3
Severe kyphosis, osteoporotic (from fractures).....	11
Severe kyphosis, Scheuermann's type.....	0

than that of the average female. The human female is one of the longest lived of all mammals.

FREMONT-SMITH: The men were much younger, were they not?

URIST: The average age was 66 years; the average for the women was 85 years.

NICHOLS: I think your group of males was unusually short-lived.

URIST: The question that arises now is whether physiologic reduction of bone density with aging or inactivity (without collapsed vertebrae), pathologic osteoporosis, and multiple spontaneous fractures are different degrees of one and the same process. Our working hypothesis

is that excessive or extensive osteonecrosis of the cortical bone of the vertebral bodies is a characteristic of pathologic osteoporosis, but we do not have sufficient data to prove it; also, we have no idea of the cause of the necrosis. Don Fareed, a medical student research fellow, is making cell counts on cortical bone and perfusing the blood vessels of autopsy specimens.

NICHOLS: Dr. Bauer has a few figures to show which complement what Dr. Urist has been talking about. Because it is clear that we do not know much about changes in biosynthesis in the etiology of this disease, we ought to take a look at our knowledge of resorption and its disturbances to see if we can learn something from the animal models now available.

BAUER: I would like to present some data on the incidence of fractures in the population of Malmö, Sweden. Figure 91 (ref. 162) shows the annual incidence per 10 000 inhabitants for fracture of the distal end of the radius in males and in females. At about age 40 there occurs a sudden dramatic rise in the incidence of this fracture in females.

Figure 92 shows that there is a distinct difference between the two sexes also as to what has caused the fracture. The figure shows the ratio of slight over severe trauma, slight trauma being defined as a fracture occurring from a fall on the floor or something similar; if more trauma was involved, then it was registered as severe. We see again a very distinct difference between the two sexes.

In figure 93 we see, in the same population, the change with age in location of fracture of the radius. With increasing age the incidence of distal fractures rose in relation to shaft fractures, and dramatically more so in females as compared with males. This again suggests that something is happening in the skeleton with age, and more so in females than in males.

Figure 94 (ref. 163) shows the incidence, in the same population, of fracture of the distal end of the radius and of the neck of the femur. There is a parallel rise in the incidence of these two types of fractures with a lag period of some 16 years.

I show the diagram (fig. 95, ref. 164) to illustrate that one cannot relate fracture immediately to fragility of bone. Fracture in general can be caused by external violence or by disease; cancer metastasis or radiation injury, for example, tend to make the neck of the femur weaker than normal. But when one is looking at data of this type, one cannot escape the conclusion that in addition to external violence and such diseases, which we can define at the present time, something is happening in the skeleton that is closely related to age, and also that this progressive age factor, fragility, is an important factor in causing certain fractures in the aged.

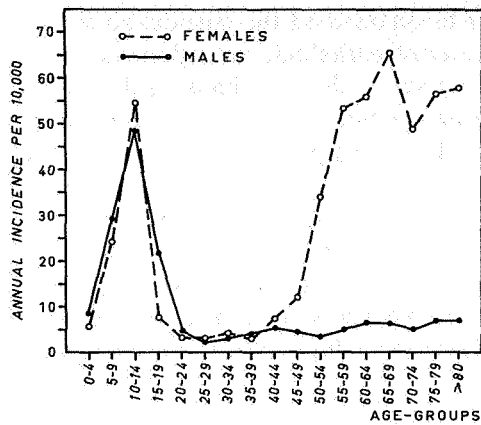


FIGURE 91. The annual incidence of fractures of the forearm according to age in Malmö, Sweden. [From ref. 162; reprinted by permission of the publisher.]

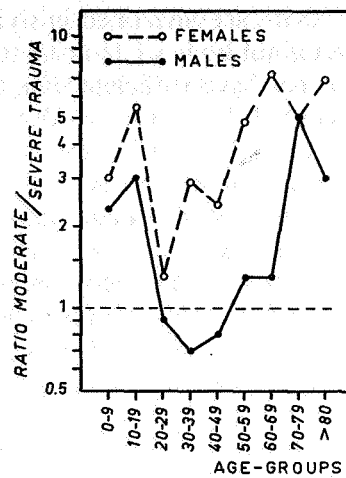


FIGURE 92. Ratio of moderate to severe trauma in males and females. The data did not permit evaluation of the degree of trauma in all cases. [From ref. 162; reprinted by permission of the publisher.]

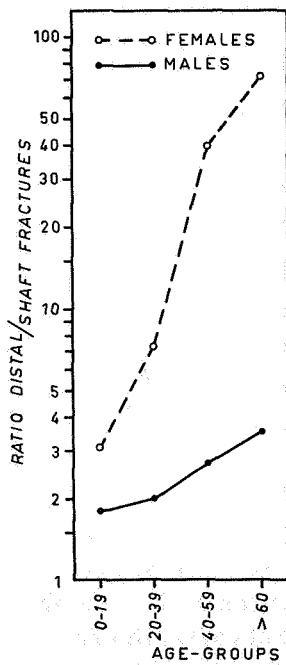


FIGURE 93. Change with age and sex in the location of fracture of the radius. [From ref. 162; reprinted by permission of the publisher.]

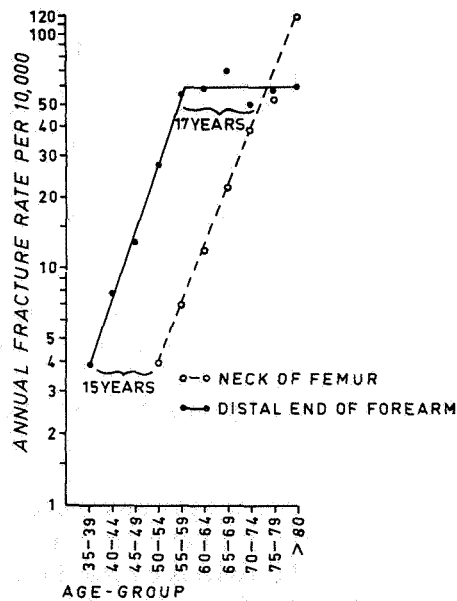


FIGURE 94. Semilogarithmic plot of age-specific rates of incidence in women of fractures of lower end of forearm and of upper end of femur. [From ref. 163; reprinted by permission of the publisher.]

If we are interested in trying to understand this age-linked fragility of the skeleton, we have to make a distinction between two mechanisms for making the skeleton fragile. One is too little bone, and the other may be, and this is relatively hypothetical, a change in the quality of the bone tissue. Unless we try to make this distinction, we will tend to overlook the possibility that fracture may occur because the skeleton is weaker than normal without there necessarily being any less bone than normal.

I submit that the epidemiologic data on fractures of the distal end of the radius in females are especially difficult to interpret solely in terms of too little bone. It is quite clear that the skeleton loses bone with age, but nobody has shown that the female at age 40 or 45 has lost enough bone to cause this rise in fracture incidence.

Therefore, I believe it is important to measure the strength of bone independent of how much bone there is.

One could then say that osteoporosis may be defined as a disease in which there is fracture from inadequate violence; one etiologic factor may be "osteopenia," meaning too little bone, and another "fragility," meaning a condition where the quality of the bone tissue has deteriorated. The question was raised: "Too little as compared with what?" Naturally, too little as compared with normal. I do not think one must show that it is bad to have too little bone compared with normal any more than one must show that it is bad to be underweight or overweight. It is true that because we can link sideropenia to definite symptoms of disease, it is beyond doubt a distinct disadvantage to have too little iron; and it is very definitely bad to have too little money, even though what is normal is hard to define. Even though "underweight" is not defined in terms of one weighing so little as to be blown into the ocean by a gale of, say, 20 miles per hour, it is possible to say that outside of two sigmas of normal, one is underweight; then one is able to determine if it is bad to be underweight. In the same way, I think one can very well talk about a condition of too little bone, even though perhaps it is difficult to measure, and even though one does not know whether it is bad to have it. I think it probably is.

NICHOLS: Then you would put osteoporosis into my category of disturbance of cell machinery; the difference being that you believe it is an acquired, rather than a congenital, disturbance; is that correct? In other words, do you think something goes wrong with the machinery so that the way in which the bone is made is wrong?

BAUER: I do not have any ideas about the etiology here.

NICHOLS: I am serious. This is a possibility.

BAUER: That is why I am so serious. I have not talked about the etiology. To give an example of what I am talking about, one could

say that perhaps the quality change comes about because of a change in polymerization or crosslinking; perhaps the collagen fibrils suddenly become tired and do not bite each others' tails as well as they should. But it is a very interesting fact that this possibility has not been discussed and even less studied.

MCLEAN: I think it should be pointed out that there is not too little bone for the metabolic functions of the skeleton, which hold up very well.

BAUER: Oh, yes.

MCLEAN: It is too little bone for the structural functions of the skeleton.

BAUER: I am trying to say that I do not know whether it is too little for metabolic function or too little for a structural function. I define "too little bone" as being less bone than normal in the skeleton, and I would like to know what structural importance this has. I am betting on the possibility that if I have less bone than normal, my skeleton becomes somewhat less strong than normal; but I keep in the back of my mind the possibility that without a quantitative change in my skeleton, qualitatively perhaps something happens which makes it more brittle.

MCLEAN: But you do agree that in osteoporosis the skeleton maintains its metabolic activity at approximately its normal level.

BAUER: Yes.

MCLEAN: It seems to me that it is important to differentiate between the inability of the skeleton to withstand strain and its ability simultaneously to keep up metabolic activity.

NICHOLS: What you are saying is that while one end of its function is OK, the other end may be in trouble.

HEANEY: But that is an assumption, and this is what I think Dr. Bauer is trying to say and has been trying to say for many years. I recall puzzling over this at prior conferences. There is too little bone in comparison with normal reference standards, and there is, associated with this, an increased liability to fracture. But it is another thing entirely to say that the too little bone causes the fracture.

BAUER: No, no. The thing is that you have too little bone. If you take, say, Dr. Urist's elderly ladies and you find that they have too little bone and they have fractures, or 26 percent of them have fractures of the spine, one should not jump to the conclusion that the reason they have fractures is that they have too little bone. It may be that there is a poor quality of bone, also.

MACDONALD: Is this not easily demonstrable by tensile-strength measurements?

CURREY: Well, there are practical difficulties. I think one could

calculate, quite reasonably, simply the reduction in the strength that you would expect from loss of bony material. I think there is no doubt that, in fact, there is also a change in the quality of the bone. The highly calcified regions are certainly centers which will shatter under impact in a way that ordinary bone would not. To separate the two, the change in the quality of the bone and the change in the amount of bone, and to separate their effects on fracturability of the whole bone is feasible, but technically very difficult. Certainly, it would be a very good thing if someone could get down to it.

BAUER: There is definite lack of correlation between attempts to test bone strength *in vitro* and the clinical evidence of bone fragility deduced from epidemiologic data. This is most embarrassing when we realize that we are talking of, by far, the most important metabolic bone disease we have. Twenty-five percent of Dr. Urist's elderly ladies have fracture of the vertebrae, and it has been shown in other series; in Denmark, for instance, about 25 percent of all inmates of homes for the aged have fractured vertebrae without knowing that they have it and without knowing that they have ever had any trauma. Fractured vertebrae is thus usually a very mild disease. But this is not so in fracture of the neck of the femur, which occurs in 20 percent of all women who reach 80. Fracture of the neck of the femur is not a catastrophe to those who have it; but it is catastrophic to society, not because these females would work very hard if they did not have fractures but because young people have to take care of them.

I submit that this is, in a way, what we are all studying; prevention of fracture in the aged is the man-on-the-moon project of bone metabolism.

NICHOLS: I agree with you, Dr. Bauer. There are some other ways of studying this problem that we ought to cover in this session.

NICHOLS: I presented the data that I did earlier because I wanted to indicate that one really cannot, from available metabolic data, make any sense out of the problem at all. I am not sure that one can do much better studying resorptive activity either. The fact is that we need some animal models, of which there are now a few. Whether these are models of osteoporosis is a matter which we might debate.

Dr. Raisz has been looking into Jenifer Jowsey's "osteoporotic" cats, and I hope he will tell us about them.

RAISZ: I would like to comment on the point about the difference between the supportive and the homeostatic functions of the skeleton. As Dr. McLean pointed out, the homeostatic function may be primary so that metabolic activity is maintained at the expense of support in some situations. I believe that the osteoporotic cat which Dr. Jowsey has been studying is an animal model for this, although I must admit



that at first I was reluctant to accept this model. While we agree that human osteoporosis may have many etiologies, I do not think we need to throw out the possibility of a common denominator. We do have an animal model, observed in many different species, for the effects of calcium deficiency. Calcium deficiency in different animals and in different growth states can produce typical secondary hyperparathyroidism or it can produce a state which is histologically typical of osteoporosis. Dr. Jowsey has been producing osteopenia in the adult cat by giving a high-phosphate, low-calcium diet; we have wondered whether this model might teach us anything about osteoporosis in man. There are two aspects of these animals which obviously require study. One is their parathyroid activity. We have been studying this activity in a number of such animals by measuring their parathyroid size and parathyroid amino acid uptake after long periods of calcium deficiency. These animals had normal fasting serum calcium concentrations. Homeostasis appeared to be preserved, while parathyroid activity was increased twofold to threefold as measured by the parathyroid activity index to which we referred earlier. This is far from the ideal measurement, and these questions will be clarified when good blood bioassays for parathyroid and other calcium-regulating hormones are readily available. Nevertheless, we wondered why parathyroid activity was increased with no change in fasting serum calcium. (We had seen small changes in fasting serum calcium concentration with diet in the rat.) This appeared to be a deranged feedback; however, Dr. Jowsey has found that these animals do have a period of hypocalcemia during the absorption of their diet. The animals are maintained fairly hungry and fed a restricted diet of meat all at one time. With this diet they get a large phosphate load and their serum calcium concentration actually falls postprandially. I have not seen any data to determine whether individuals with osteoporosis or other serious bone disease have any derangement in the stability of their serum calcium concentration; possibly Dr. Copp can explain this. Do some patients with osteoporosis or other chronic bone disease have instability of the serum calcium concentration, even though a normal homeostasis is maintained most of the time?

COPP: We found the diurnal fluctuations in plasma calcium concentration in osteoporotic individuals to be small and within normal limits.

NICHOLS: We too have done some calcium and EDTA infusion tests on osteoporotic patients and can find no differences from normal.

RAISZ: Another thing which Dr. Bélanger will comment on, is the dead-cell problem. Apparently the dead-cell incidence is high in osteoporosis and high in the various forms of that thing which ranges between nutritional secondary hyperparathyroidism, which is what Dr.

Bélanger was studying, and that thing which Dr. Jowsey calls calcium deficiency osteoporosis. I think we ought to look at these parameters more in these animals and in man.

COPP: I would like to make a comment here, because in young phosphate-deficient rats you get the same picture (ref. 165). Mineral is lost rapidly from the skeleton to provide phosphate essential for the soft tissues. They have very severe osteopenia and die because of collapse of the demineralized rib cage.

NICHOLS: There is another animal model—heparin osteoporosis—which is useful because it avoids the whole issue of hyperparathyroidism, which is a special disease but may well be the cause of the osteoporosis in the models cited so far.

BAUER: I can add that if you feed tigers on tiger hearts, they will develop severe osteopenia; or cats on beef hearts, which is a more common type of experiment.

NICHOLS: Some time ago a man was referred to us because the severity of his osteoporosis prevented his working; this man also had a coronary artery disease for which he had been receiving large doses of heparin for a long time, and it was quite well controlled. A variety of things suggested that his osteoporosis and his heparin therapy might be related (ref. 166), and this suggestion led to the animal model I would like to talk about. Figure 96 is a roentgenograph of the spines of two rats, one of which was kept anticoagulated with heparin around the clock for a month by John Asher in my laboratory. If you stretch your imagination, the one on the right looks a little less dense. As some of you know, Tourtellotte in Philadelphia, using sulfated low-molecular-weight dextran, produced more striking changes. I think his drug is probably a better agent than heparin because it induces less bleeding, but this is a peripheral problem. The point of importance is that the probable mechanism involved in the heparin-induced effect has now been worked out (ref. 167).

If you remember my diagram outlining the biochemistry of the bone cell, lysosomes containing collagenase were mentioned. These, it turns out, are lysed by heparin *in vitro* as is shown in figure 97. Heparin at 50  $\mu\text{g}/\text{mg}$  has little effect, but at 2500 there is clearly release of collagenase from the large-granule fraction of bone-cell homogenates which contain these lysosomes. Whether there is truly an inhibition at 10 000  $\mu\text{g}/\text{ml}$ , I am not sure.

Since, from these observations, it appeared that heparin releases the latent collagenase in bone-cell lysosomes *in vitro*, we wondered whether the changes in bone density in chronically heparinized patients or animals might be related to some effect of the heparin on the stability of lysosomes in their normal location in cells *in vivo*.

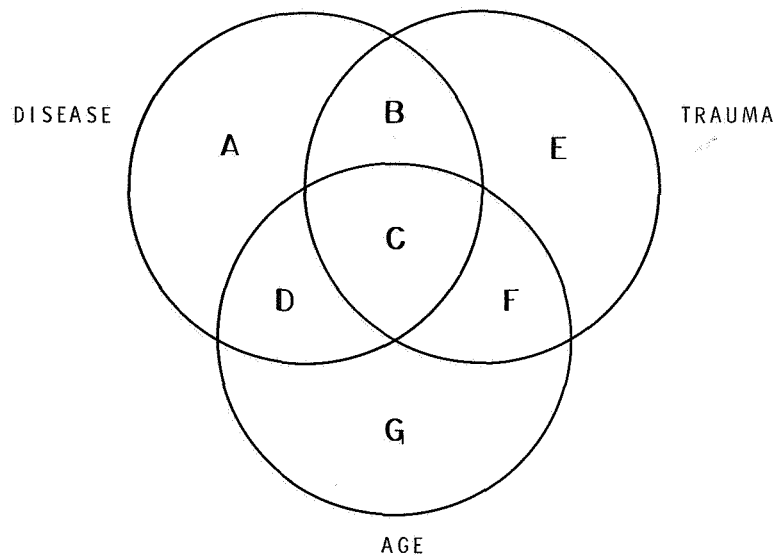


FIGURE 95. Diagrammatic representation of etiologic factors in fractures of the proximal end of the femur. [Adapted from ref. 164; reprinted by permission of the publisher.]

The results of our investigation of this possibility are illustrated in figure 98. Two sets of data are shown for lysosomes derived from bone of normal and heparinized animals. Saponin-induced activity is equivalent to the total activity present in the fraction, while "spontaneous" activity is that released spontaneously during incubation, presumably because of lysosome leakage or rupture. The ratio between the two can be considered an index of the relative resistance of the lysosomes to mechanical trauma. Simple inspection shows that heparin treatment increases total collagenase activity and the relative as well as the total amount released spontaneously. In other words, the bone-cell lysosomes in heparin treatment are more easily lysed (ref. 168). Since increased collagenase goes with increased resorption (ref. 169), these data give us an explanation for this type of osteoporosis in which no change in biosynthetic rate occurs. In contrast, one cannot demonstrate a change in the stability of the lysosome at all in parathyroid-treated animals, although one can demonstrate more total collagenase activity in the lysosome fraction.

So this is another animal model which is now available for study. This brings us to considering how bone is resorbed. Dr. Bélanger has some figures of what happens in osteolysis, a phenomenon which may hold some keys to understanding osteoporosis.

BÉLANGER: I wonder if you would like to see some figures which demonstrate the phenomenon that I mentioned a moment ago and how this fits into the animal model.

FREMONT-SMITH: Yes, by all means.

BÉLANGER: "Osteolysis," an old word which we find in dictionaries, but with various definitions, we would now like to reserve for an activity which takes place in the depth of the bone and an activity which is, it appears, related to the osteocyte at the time when this osteocyte acquires its maturity.

This sort of activity can be seen in trabecular bone as related to the center of the trabecula, and it is manifested by an enlargement of the lacuna.

When we do autoradiographs of thymidine-labeled bone we know that the thymidine appears first in the precursor cells which are outside the bone trabeculae. Then it passes out into the osteoblasts, then to the osteocytes of small size which are near the surface, then into the large osteocytes which are in the center of the trabecula, and finally this radioactive label of DNA disappears completely.

The time required for these labeled cells to go from the osteoblast stage to the last stage of mature osteocytes, of course, varies from one area to another of the body and varies with the age of the animal. The younger the animal, the faster this takes place. In the vertebrae of a rat, this lifespan of a bone cell is approximately 4 days.

YOUNG: How do they move through bone in 4 days?

BÉLANGER: Figure 99(a) is what we call an alphasradiograph of soft tissue. In this case, we have removed the mineral and have obtained, through bombardment with alpha particles, a density image of the tissue. The periosteum and the bone trabeculae are shown, and we can see now that the density of the organic matrix is far greater at the periphery than at the center of the trabecula, which is contrary to the degree of mineralization. We recognize this large lacuna in the center of the trabecula.

Now, as Dr. Young asks, "How do things 'move'?" We think that they move because large osteocytes, before they die, are actually perfectly capable of producing proteolytic enzymes which destroy the matrix around them and release the mineral, so that there is a continuous new stream of bone material from the surface to the center of the trabeculae as these cells die out, as the matrix is broken down and as the mineral is released. And so the bone which is here at any one time is not the bone which was there half an hour earlier.

Just to show the affinity of these effects, here is some material that I have obtained from Dr. Copp. Figure 99(b) represents a parietal bone of a sheep in which we can see these spaces, and then lacunae

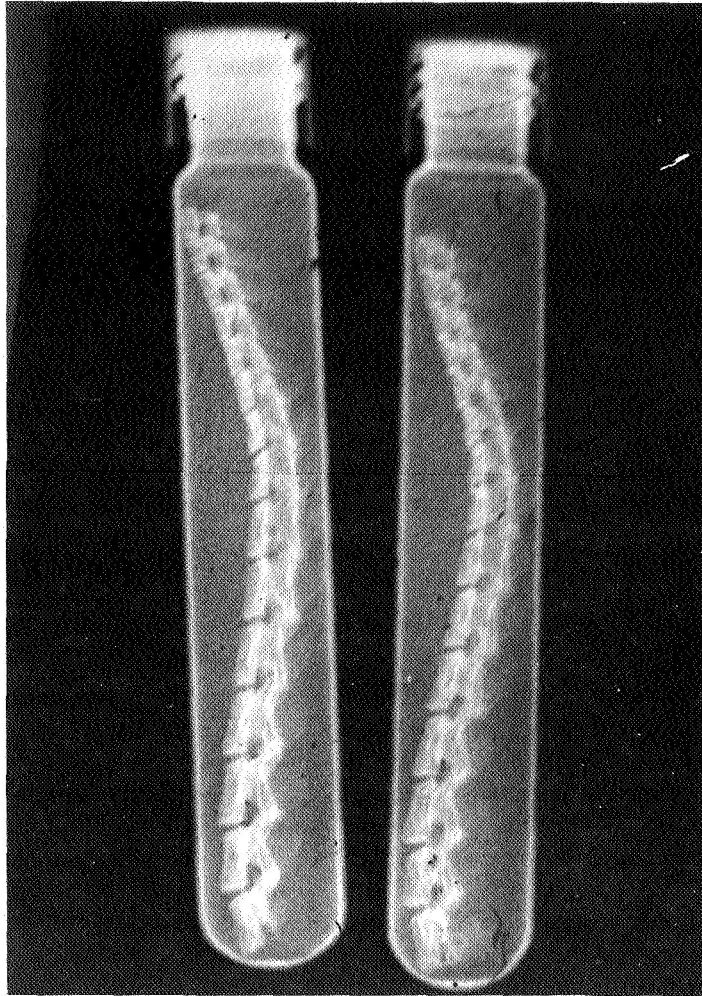


FIGURE 96. Roentgenograph of spines from two rats: Control (left) and anticoagulated with heparin (right).

with osteocytes which are either small or large in a normal sheep.

Figure 99(c) shows bone from a sheep that had been infused with EDTA intravenously for a period of 4 hours. We see again a very large number of large lacunae.

In figure 100, corresponding to the presence of these large lacunae, we also can see, after toluidine blue staining, that there is an increased amount of metachromatic material, both inside the lacunae and outside in the matrix which surrounds these cells. This was the first phenomenon which we observed, and we attached considerable importance to the presence of these acid mucopolysaccharides, thinking that they had

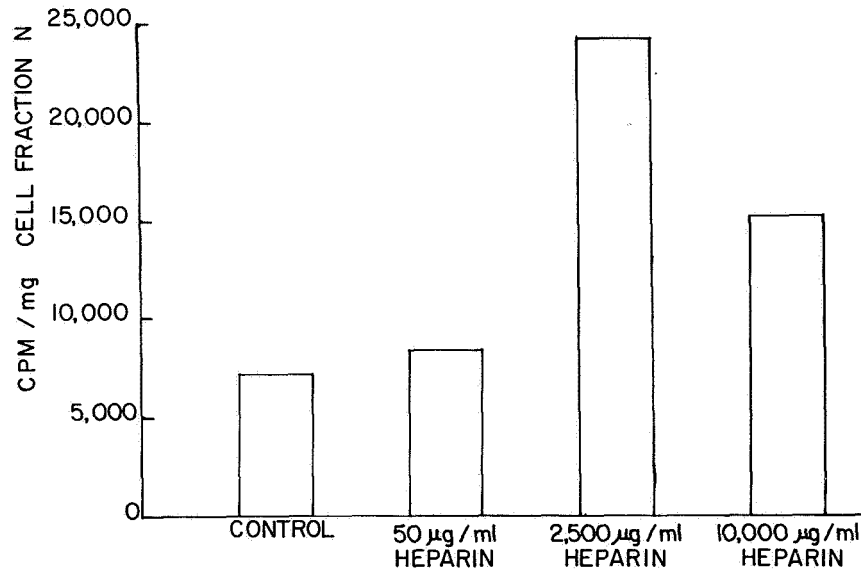


FIGURE 97. *In vitro* effect of heparin on large-granule collagenolytic activity.

a great deal to do with changing the pH level and releasing the mineral.

CURREY: Have you got a control picture to that one?

BÉLANGER: No; I do not have.

CURREY: This is rather the same point Dr. Pritchard brought up.

BÉLANGER: This has been in the literature so long I did not think anybody would be interested; this has been in the literature for many years.

BAUER: It still may be true, though.

BÉLANGER: Oh, yes; right.

PRITCHARD: Dr. Bélanger, there is another possible explanation. The first bone laid down under the periosteum, or the first bone laid down in the metaphysis, is of different character and quality from the bone laid down a few days later. The first bone laid down has irregular, coarse fibers and big cells. The later bone that buries the first bone has finer, more regular fibers and small cells. Now, how can one be sure that this is a dynamic process and not just two kinds of osteocytes?

BÉLANGER: This is an adult animal, and in the work which we did with labeled cells we know that this bone has been totally replaced in 3 or 4 days.

YOUNG: Figure 101 shows a region of woven bone and a portion of lamellar bone. The question that some of us raise is whether those cells in the center of the trabeculae you showed, those cells that have

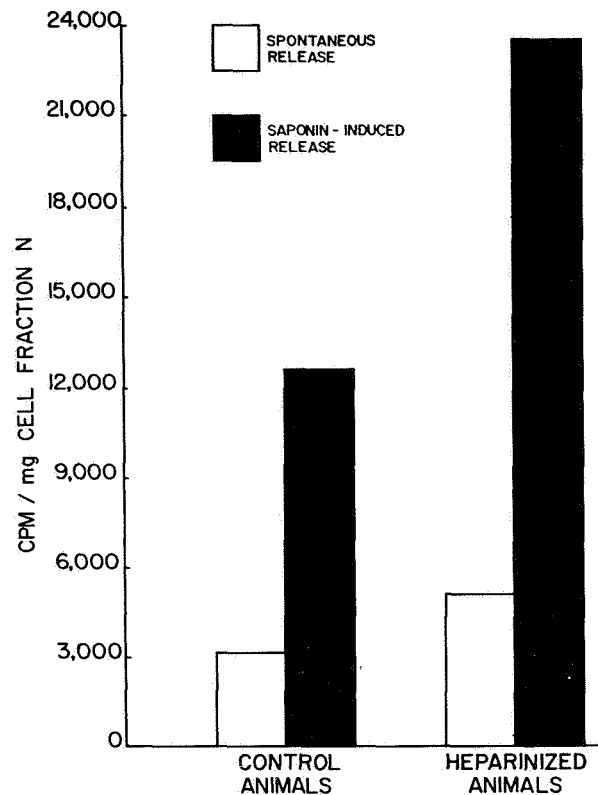


FIGURE 98. Large-granule collagenolytic activity.

enlarged lacunae and are stained differently, simply may not represent the larger osteocytes characteristic of woven bone, rather than osteolysis.

BÉLANGER: I do not worry too much about that particular point, because we later came up with a more important observation. This factor may be more significant because it may contribute to the destruction of the organic part of the matrix. We took photographic plates that have been blackened inside and processed. Over these we put a drop of trypsin and placed pieces of different types of tissue in a buffer of pH 7 on the film. We put a piece of pancreas, sections of fresh bone, and a section of bone that had been fixed in formalin for 5 minutes on the film. The pancreatic tissue made a hole in the film. The formalin-fixed bone did nothing. The fresh-bone sections made a considerable hole in the gelatin, tending to show, I believe, that this little piece of bone actually contains an enzyme capable of doing the same thing as the pancreatic tissue or trypsin.

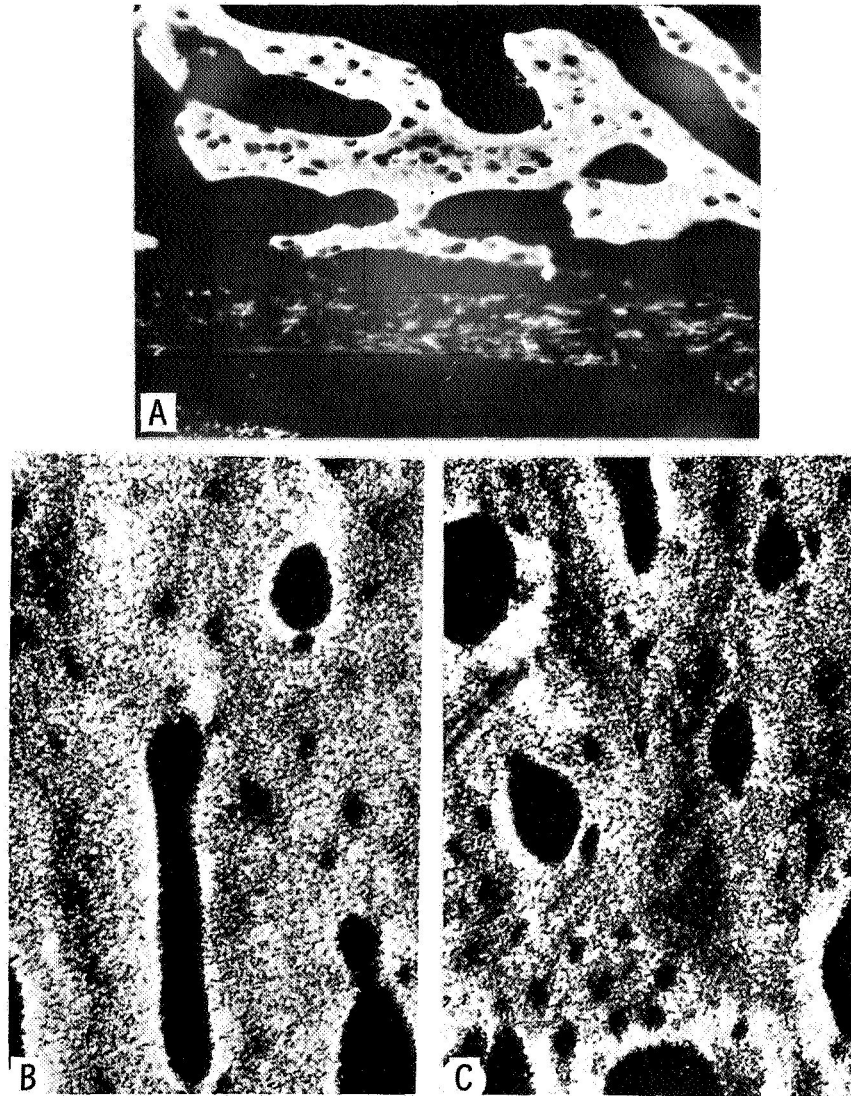


FIGURE 99. Alpharadiographs of demineralized bone sections. (a) Dog vertebra: Note the dense matrix of trabeculae as compared with periosteum (bottom); note also the enlarged lacunae and osteolysis in the trabecula (center). 114 $\times$ . (b) Parietal bone of a normal sheep: Areas of low density in the matrix are seen in the vicinity of the larger lacunae. 182 $\times$ . (c) Parietal bone of a sheep infused with EDTA for 4 hours: The large lacunae are more numerous and confluent in some areas. 182 $\times$ .



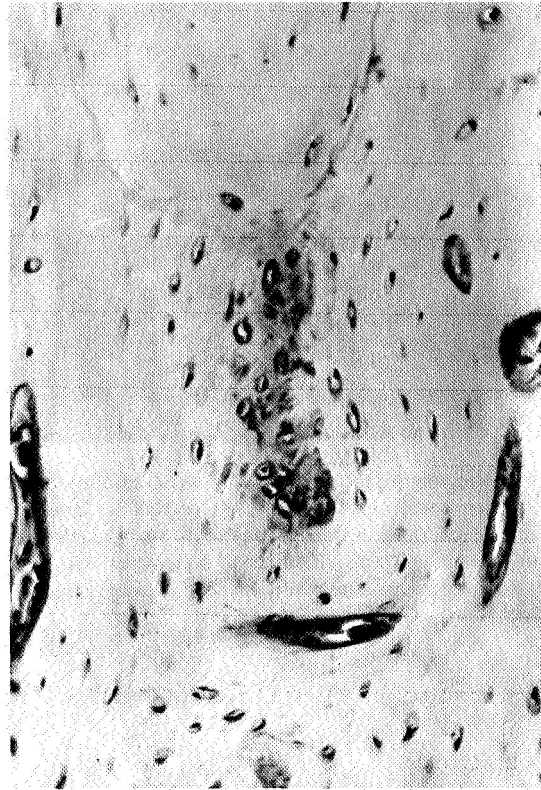


FIGURE 100. A portion of the tibial diaphysis of a horse on a high-phosphate diet for 7 weeks. Note the large osteocytes surrounded by metachromatic matrix. 136X.

When we do this sort of thing on actual section it is possible to pinpoint the site in the bone where this proteolytic enzyme, or enzymes, come from.

URIST: Is this a histochemical preparation that is analogous to what Jerome Gross and George Nichols observed with quantitative methods on tissue cultures and explants?

BÉLANGER: Yes; that is correct. This is the so-called reaction of Adams and Tuqan (ref. 170), which was devised in England and makes use of the same principle of breaking down gelatin.

PECK: The ability to break down gelatin is the property of many, many enzymes. The ability to break down native collagen is a prop-

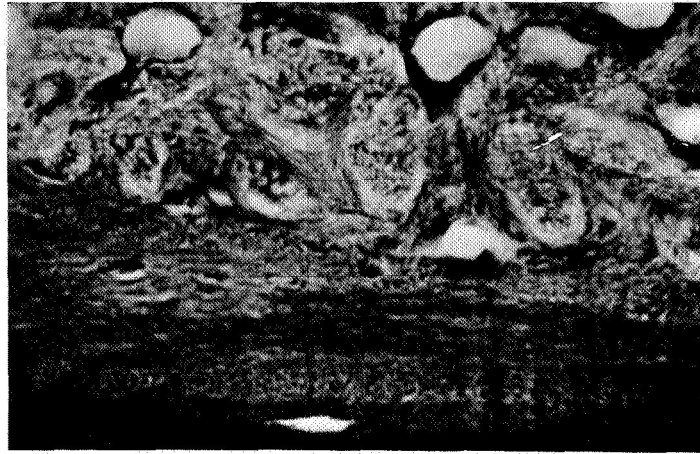


FIGURE 101. Region of parietal bone from a 26-day-old rat. Note that in the woven bone (top) the osteocyte lacunae are large and closely spaced, and the intervening collagen bundles are irregularly arranged. In the lamellar bone (bottom), the lacunae are smaller and farther apart, and the collagen bundles are more densely packed in regular array. Gomori silver stain. 1250 $\times$ .

erty of collagenase; this is what Dr. Gross and Dr. Nichols have demonstrated.

BÉLANGER: Of course.

URIST: I understand that this is not collagenolysis, which is something else. I also understand that when you use the term "collagenase" you are talking of an enzyme that breaks protein down to amino acids, but when you are talking about collagenolytic enzymes it is not necessarily so.

NICHOLS: When you talk of collagenase at present, you are talking about an enzyme which works to break down a native collagen molecule (the "nativeness" of the collagen molecule needs definition in its own right) into reasonably large peptides. The further degradation of those peptides probably depends, as far as we understand the process at the moment, on a peptidase; but the question of whether this is the same enzyme as these more nonspecific proteases, which are clearly present in the cell and are active both at neutral pH (ref. 169), remains unanswered.

BÉLANGER: When you do this sort of thing on the bone section, this is actually a unicellular response which we get in the center of trabeculae where each of these large osteocytes is located (fig. 102). There is a ring of tissue destruction immediately surrounding the lacunae there.

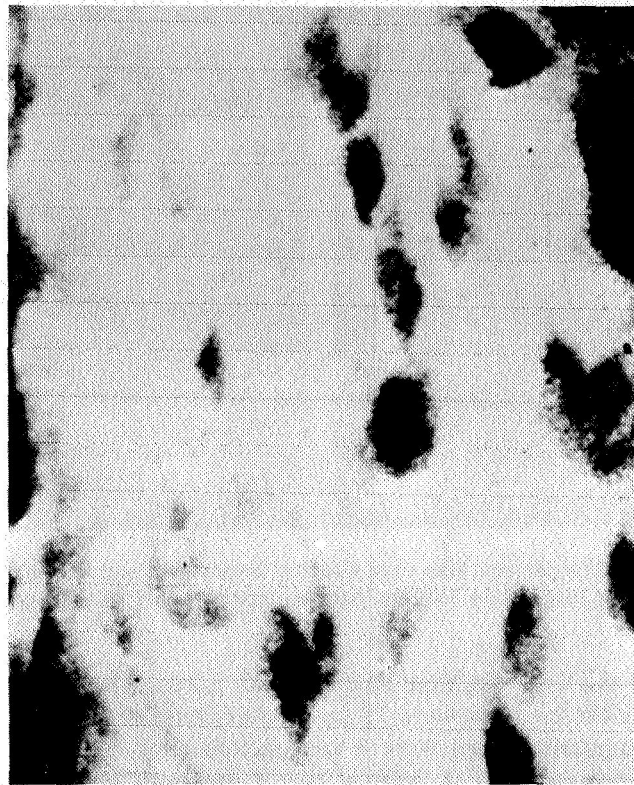


FIGURE 102. Microradiograph of an undemineralized section of the femur of a frog. Note the low density of perilacunar bone. 520X.

Figure 103 is a gross type of operation that we made last summer, using frog bones. These are femurs and humeri from two tadpoles that had been injected with a single dose of 5 units of PTE. You can see by the size of the reaction which we have recorded on the film that, indeed, PTE has increased considerably, on the average, the production of protease by these pieces of bone. If we drew these out, cut the drawings and weighed them, the proportional increase could be obtained; this has been found to be sixfold.

That this might also be related to the production of collagenase—and this is where we join our friends Gross and Lapière in Boston—is suggested by the fact that these tadpoles have been injected with parathyroid hormone. We have measured the total length of the animal after 1 day of parathyroid-hormone treatment and compared it with controls. Starting with a length of 12 centimeters for the total tad-

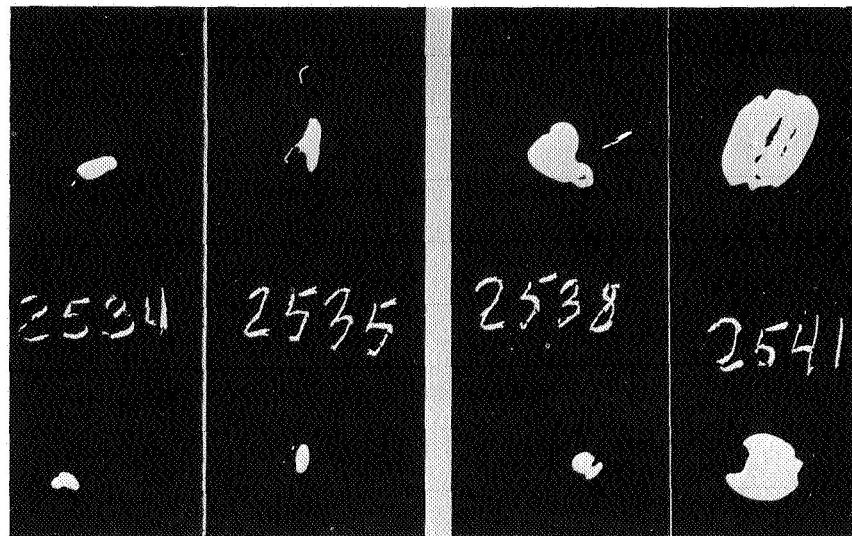


FIGURE 103. Protease reactions from bisected femurs (top) and humeri (bottom) of normal tadpoles (two on the left) and tadpoles treated with PTE (two on the right).

pole—and this is at the time when the tail is resorbing very rapidly—after 1 day the controls were 11 centimeters long and the parathyroid-hormone-treated animals were 9 centimeters long. So it is from the tails of these tadpoles, I believe, that Gross and Lapière (ref. 171) isolated collagenase. I think that the collagenase is affected by the system, as well as the protease, which we have demonstrated in the bone cells.

If you would be interested to see the cells that produce this enzyme, we have some electron micrographs.

NICHOLS: I think it should be pointed out in confirmation of Dr. Bélanger's ideas that Gilbert Vaes, working in Gaillard's laboratory, has measured the release of various bone lysosomal enzymes in tissue culture and found 8 to 10 times more enzyme when parathyroid hormone was added to the culture medium. Moreover, the total enzyme content of the culture medium after incubation was far beyond the enzyme content of the tissue originally transplanted, so that this phenomenon is clearly representing a stimulus of biosynthesis and not just increased release of preformed material (ref. 172).

BÉLANGER: These are micrographs from 2-day chick embryonic tibias, a system which actually replaces itself very rapidly. First I will show some figures belonging to Professor Baud (ref. 173) from Geneva and published in 1962, where he showed that in bone he could see osteocytes in what he called smooth lacunae, and these were at the

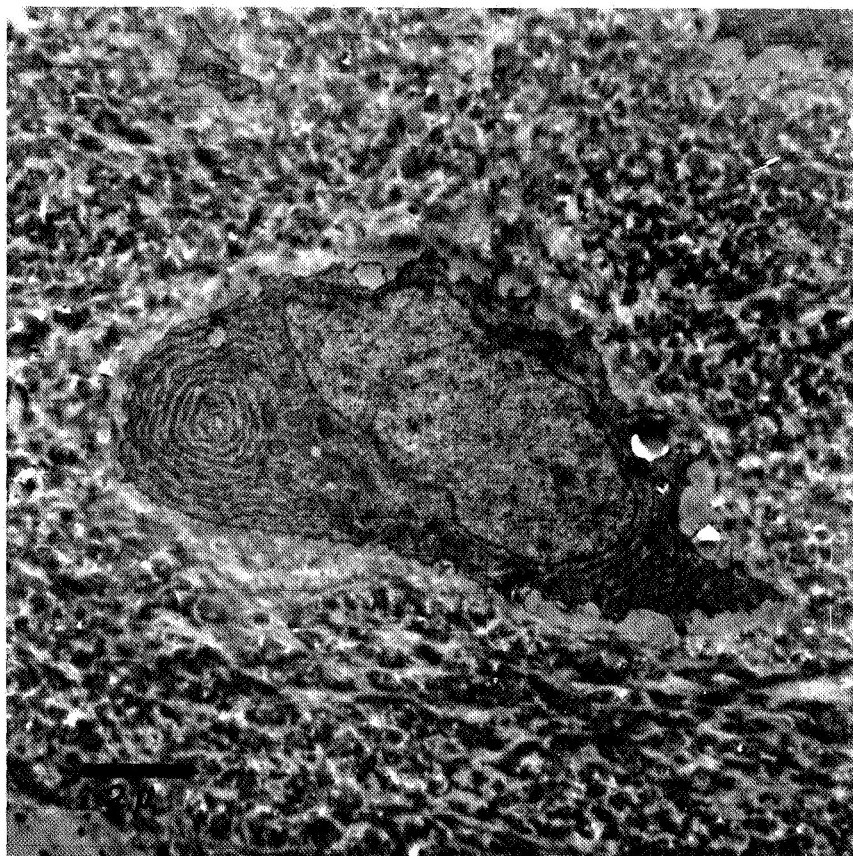


FIGURE 104. Peripheral osteocyte from the tibia of a 2-day-old chick embryo. The cell is closely encased by the matrix. Endoplasmic reticulum, ribosomes, and mitochondria are prominent features.

surface of the bone. They were osteocytes that were embedded in much larger lacunae in which the wall appeared to be rough.

In our own chick material (fig. 104) a young osteocyte is located near the surface. Actually, we can see that it fits quite neatly inside its lacuna and that it has most of its cytoplasm on one side of the nucleus, as an osteoblast might also have. We can see mostly endoplasmic reticulum, mitochondria, and some people can see a bit of Golgi complex even at this low power.

When we come to the osteocytes that are located deeper inside the trabeculae (fig. 105), we can now see large, osmiophilic vesicles. The size of the lacuna is also increased. Finally, the largest of these osteocytes, which we find in confluent lacunae in the center of the trabeculae, contain a larger number of these osmiophilic vesicles in their cytoplasm.

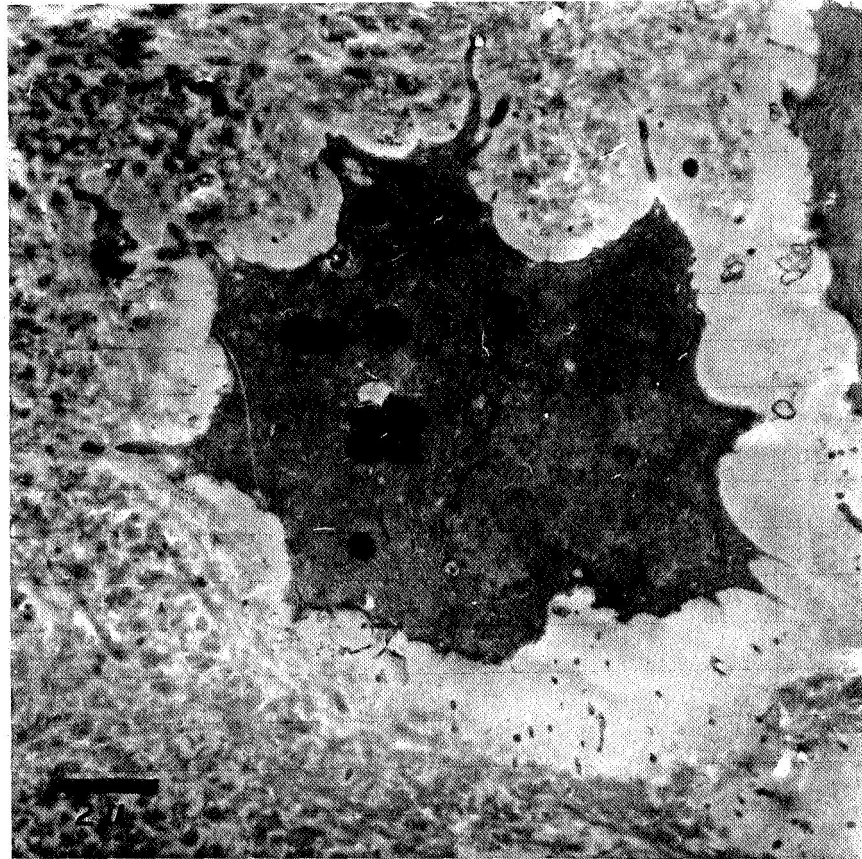


FIGURE 105. A centrally located osteocyte from the same specimen as in figure 104. The lacuna is wide and apparently confluent with an adjacent one. Vesicles containing osmiophilic material (lysosomes?) are prominent.

Some people say I should call them "lysosome-like" because I do not know whether or not they are lysosomes; they look like lysosomes.

If we give parathyroid hormone to some of these chick embryos, putting PTE in the yolk, 2 units per gram for a chick of 7 grams in weight, more or less, for 24 hours, this is what we see (fig. 106). The osteocytes near the surface apparently mature very rapidly. Now the endoplasmic reticulum has become enlarged, and there are cisternae, such as described by Baud (ref. 173). The osmiophilic vesicles which hardly exist in the young osteocyte become fairly numerous after parathyroid hormone, and we even begin to see a structure like this, which some electron microscopists tell me is an autophagic body. This apparently is related to cell digestion from enzymes which issue from some of the osmiophilic vesicles.



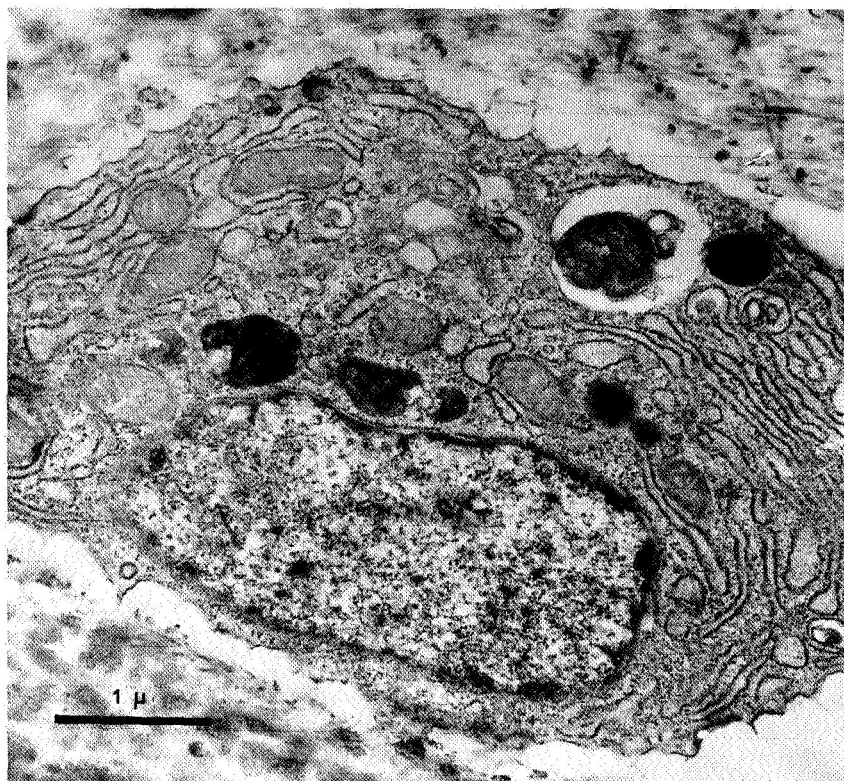


FIGURE 106. Peripheral osteocyte from an 11-day chick embryo treated with PTE. Enlarged cisternae and numerous pinocytotic vesicles are visible; more numerous osmiophilic vesicles are also present; some appear to have been released into the lacuna.

So it is apparent that parathyroid hormone has increased the maturity of the cells, and has increased—as part of the maturity of the cells—the production of the osmiophilic vesicles which might be related to the production of protease. If this goes on for a time, the whole cell is destroyed, and we are left with an empty lacuna.

NICHOLS: I would like to say something about fluoride. I have one more figure about another osteoporotic patient.

We need to learn more about fluoride and its effects, because it is possible that we might learn how to treat osteoporosis even though we do not know what it is. Figure 107 shows roentgenographs of the lumbar spine and pelvis of a woman who came to me because she had developed acute pain in her back one day when she jammed on the brakes of her car. The picture on the left was made shortly after this episode when she was experiencing considerable pain from a crushed vertebra, and had obvious osteoporosis. One year later, she had the

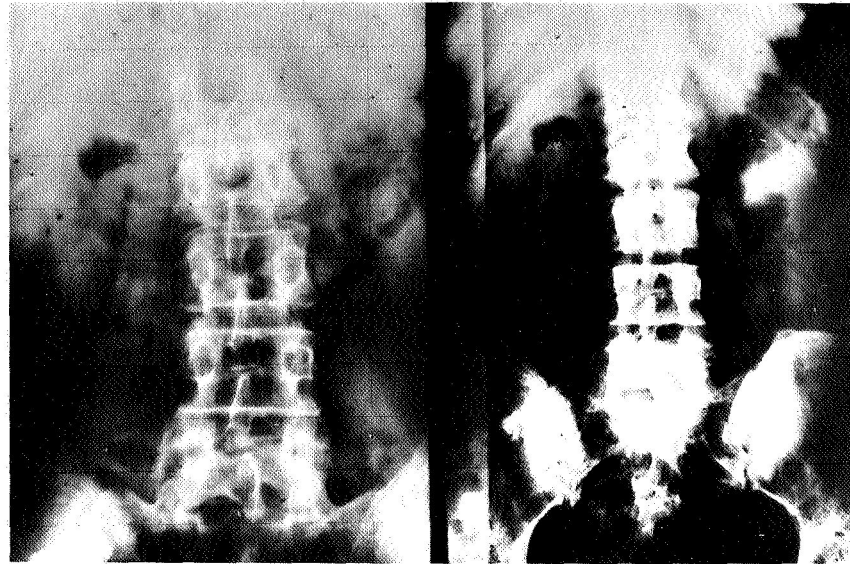


FIGURE 107. Roentgenographs of lumbar spine and pelvis of a 53-year-old female with osteoporosis (left) and after fluoride treatment (right).

picture on the right, which shows considerable thickening of the trabeculae in her vertebra and pelvis.

The woman was 53 years old. The clinical data which were found at that time are shown in the top half of table XXII. Serum calcium concentration was 4.6 mEq/l; alkaline phosphatase was normal, and urinary hydroxyproline excretion, which may or may not be an index of the rate at which collagen is destroyed in the body, was actually lower than normal of 28 milligrams for 24 hours. Metabolic studies of her bone biopsy showed normal lactate production and incorporation of glucose into collagen and cells. Proline incorporation into collagen was normal, but cell labeling was a little high and  $O_2$  uptake was just above the normal range as we have defined it.

She was started at this point on 100 milligrams of sodium fluoride per day. About 5 months later she was completely symptom free and she has never had any pain since, nor does she have any evidence of any new crushed vertebra at this point. It took about 10 months for her to develop any roentgenographic change that we could recognize. When we did we biopsied her again.

This was 11 months after the first one. Serum calcium and so forth were normal. She did, however, show elevations of serum alkaline phosphatase activity and urinary hydroxyproline excretion. Bone metabolic data showed the same  $O_2$  uptake but reduction in lactate production. The striking changes are in the measurements which re-



TABLE XXII  
SKELETAL METABOLIC CHANGES IN IATROGENIC FLUOROSIS  
[Female—Age 53]

Clinical data	July 1964 osteoporosis	June 1965 osteoporosis + fluorosis	Normal
Serum Ca, mEq/l.....	4.6	4.9	4.5–5.2
Serum F, mmol/l.....	1.3	1.2	1.0–1.5
Alkaline phosphatase, B–L units.....	1.2	2.4	0.5–2.2
Urine hydroxyproline, mg/24 hr.....	15	40	24 ± 4.9
<i>Bone metabolic data per mg DNA:</i>			
Oxygen uptake, $\mu$ moles.....	0.92	0.91	0.47 ± 0.22
Lactate production, $\mu$ moles.....	1.17	0.72	0.79 ± 0.29
Glucose incorporation, m $\mu$ moles:			
Cells.....	551	901	320 ± 170.0
Collagen.....	4.60	20.7	4.00 ± 0.92
Proline incorporation, m $\mu$ moles:			
Cells.....	66.0	64.1	23.4 ± 7.84
Collagen.....	0.19	0.79	0.24 ± 0.09

late to matrix biosynthesis. Glucose incorporation is now three times the normal value. The ratio of glucose to collagen is five times normal and the incorporation of proline label into collagen and cells is similarly increased. In other words, these data show that the roentgenographic changes we call fluorosis are clearly reflected by increased bone-cell metabolism—especially in the area of bone collagen synthesis. This is important because it indicates that one can do something about this disease. One can substitute another disease for the one which is troubling the patient, a disease which is, relatively speaking, asymptomatic.

The question that we do not know the answer to as yet is, “What happens when fluoride is stopped?” This patient has been off fluoride now for 4 months. Her alkaline phosphatase is back down to the normal range, but whether this means that she is going to revert to her previous osteoporotic state I do not know. Time will have to supply the answer. She is, by the way, our one patient who has agreed to have a third biopsy.

HOWELL: Is that not a calcified paravertebral ligament on this patient's spine?

NICHOLS: Yes.

COPP: Is there any relationship between heparin-induced osteoporosis and the fluoride type, and vice versa?

NICHOLS: There appears to be, in fluorosis, a stimulation of the biosynthetic machinery to make more matrix. What we do not know is whether there is a simultaneous specific inhibition of the biosynthesis of collagenase or other catheptic enzymes. This remains to be looked into. Unfortunately, we do not have an analysis of collagenolytic activity prior to sodium fluoride in this woman.

The cause of the stimulation of matrix synthesis is not known either. In animals we have some observations which show that one can reproduce this biosynthetic stimulation. Dr. Peck showed data last spring indicating that collagen synthesis is inhibited by fluoride, and we have had the same experience at certain dose levels. On the other hand, if the animal lives long enough, the corner seems to be turned and a relative stimulation seems to begin. Dr. Peck, do you have a comment?

PECK: We were giving large doses of fluoride in drinking water to weanling rats, and the situations are obviously not quite comparable (ref. 153). Significant inhibition resulted, which was dose related. Our greatest effects occurred about 50 ppm for about 2 weeks to 1 month in a weanling rat. That is really a lot of fluoride.

URIST: Dr. Nichols, you have done a beautiful job. We will now adjourn.

## GENERAL SESSION I

### Discussion Leaders:

DR. MARSHALL R. URIST  
DR. FRANKLIN C. MCLEAN

MCLEAN: To avoid any misunderstanding, I would like to expand on the discussion of the previous session concerning the effect of parathyroid extract in the rat. The following figures illustrate sections of bones taken from rats that received 1000 units of parathyroid extract. This is old stuff, but it is so old I think it has been largely forgotten, so I can bring it to life again.

Figure 108 shows a section of a tibia from a 6-week-old normal control rat with about the normal amount of primary spongiosa and very active osteoblastic activity. Figure 109 shows the tibia 9 hours after PTE administration. At greater magnification, pyknotic nuclei of osteocytes may be seen and also the cluster of osteoclasts around the spicules of bone that are undergoing resorption. Figure 110 is a photomicrograph 24 hours after PTE administration, and the primary spongiosa has virtually collapsed and is amputated from the secondary spongiosa; there has been very widespread and very active resorption in the area of the primary spongiosa.

Figure 111 shows the tibia 4 days after PTE administration; note the amount of reconstruction and new bone. All of the bone in the spongiosa is new bone that was formed after the initial phase of resorption, which almost destroyed the spongiosa.

Figure 112 is a higher magnification of figure 111, and there is construction of new bone with very active osteoblasts throughout. This is what used to be called hyperostosis, and the point that I made previously was that this reaction has been observed only in the rat. I also made the point that by controlling the dosage and the timing, either an osteoblastic or an osteoclastic reaction may be produced. With smaller doses of parathyroid extract—and remember, we are dealing with Lilly's parathyroid extract—it is possible to arrive at this stage of hyperostosis without ever going through the stage of resorption and osteoblast formation.

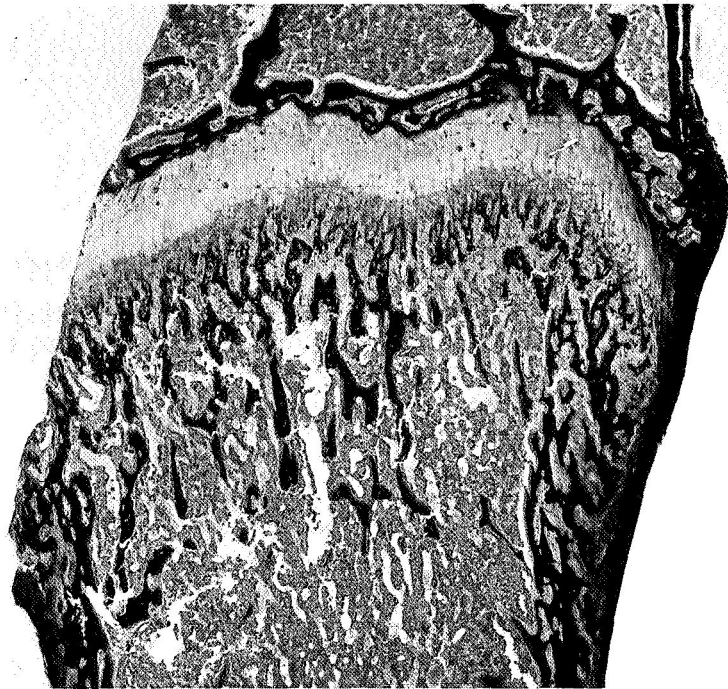


FIGURE 108. Photomicrograph of a longitudinal section of a tibia from a normal rat, approximately 6 weeks old. Zenker-formol fixation; HEA stain. 16 $\times$ .

RAISZ: Do those small doses cause hypercalcemia?

MCLEAN: With doses smaller than those that will produce hypercalcemia, one can produce hyperostosis, or the growth of new bone. Again, I emphasize that this is in the rat; since the rat does react this way, one can account for Dr. Young's ability to produce at will, by proper timing and proper dosage, osteoclasts or osteoblasts from osteoprogenitor cells.

NICHOLS: Were these repeated doses, Dr. McLean?

MCLEAN: To achieve the osteoblastic reaction without the osteoclastic reaction, small doses of around 50 units must be given daily over a period of 3, 4, or 5 days. This will produce a lot of new bone, but there will be no stage of osteoclastic resorption.

NICHOLS: I ask because we conducted a somewhat similar experiment and found that there is indeed a period lasting a day or two when there is a fall of collagen biosynthesis from proline. Later, this effect is reversed and an increase can be shown (ref. 152). Our data are very similar to yours in that respect. We had, however, some degree of hypercalcemia in these animals. Also we found an increase by the

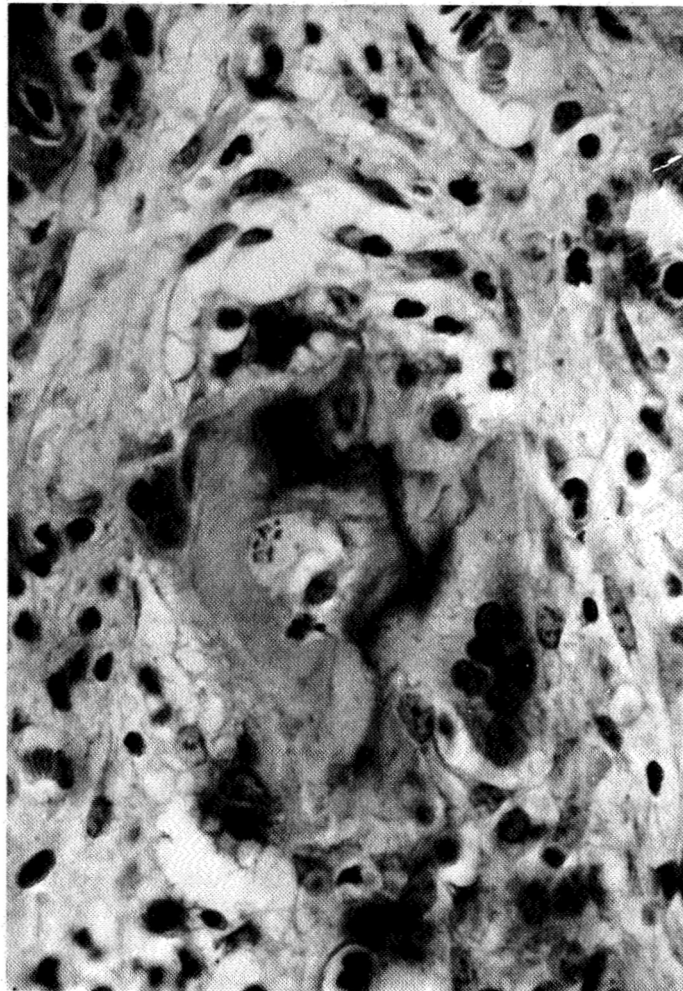


FIGURE 109. Photomicrograph of a section of a tibia from a normal rat 9 hours after administration of 1000 units of parathyroid extract. Note the pyknotic nuclei of osteocytes and the cluster of osteoclasts around the spicules of bone that are undergoing resorption. Zenker-formol fixation; HEA stain. 800 X.

tissue in lactate production which starts right away at day 1 and gradually builds up to its final level.

MCLEAN: In the intact rat, if the dose is small enough, osteoblastic activity will appear in new bone formation without any stage of resorption.

BÉLANGER: Dr. McLean, do you know whether this new bone which was formed under these conditions is normally mineralized or is it undermineralized bone?



FIGURE 110. Photomicrograph of a longitudinal section of a tibia from a normal rat 24 hours after administration of 1000 units of parathyroid extract. The primary spongiosa has collapsed and is amputated from the secondary spongiosa. Zenker-formol fixation; HEA stain. 16 $\times$ .

MCLEAN: In the stage that is shown in figure 112, it is undermineralized. It has not had time to catch up with the very rapid formation of bone.

BÉLANGER: You would not call this osteoid?

MCLEAN: No; it is not osteoid. It is partly mineralized.

PRITCHARD: Can anyone tell me exactly what calcitonin does to a bone-cell population?

COPP: Aliapoulios and Munson (ref. 174) have suggested that you may get stimulation of bone formation as well as inhibition of resorption.

PRITCHARD: Has the actual cell population been studied histologically?

COPP: No.

NICHOLS: We have some data on the response of the tissue, again in terms of the proline incorporation into collagen. This is increased, although it is not the first response seen after administration of the hormone. I cannot tell you whether this happens in parathyroidectomized animals.



FIGURE 111. Photomicrograph of a longitudinal section of a tibia from a normal rat 4 days after administration of 1000 units of parathyroid extract. Note the amount of reconstruction and new bone. Zenker-formol fixation; HEA stain. 16 $\times$ .

TALMAGE: With reference to different effects produced by varying the dose of parathyroid hormone, I was with Professor Gaillard a couple of years ago and we were doing organ culture—I must be very careful to talk about organ culture instead of tissue culture—of radii taken from embryonic mice. It was observed that if a radius was cultured from 18-day embryos with 0.01 unit of PTE for a 24-hour period, what looked like osteolysis occurred. There were almost no osteoclasts formed. However, in bone already formed in the embryo, the lacunae became larger, indicative of resorption. If the other radius taken from the same embryo was cultured with 0.1 unit, in just a matter of 8 hours large osteoclasts were formed. This, then, is a differentiation because of the dose. I believe the difference is based on the fact that there are two different types of action of parathyroid hormone in bone. One effect of the hormone is in rearranging or reorienting the DNA-RNA relationship. Normally this effect is only seen in mesenchyme cells, but if a large dose is administered not only the mesenchyme cells are affected, stimulating the production of osteoclasts, but also the osteoblasts are affected. At these high doses the

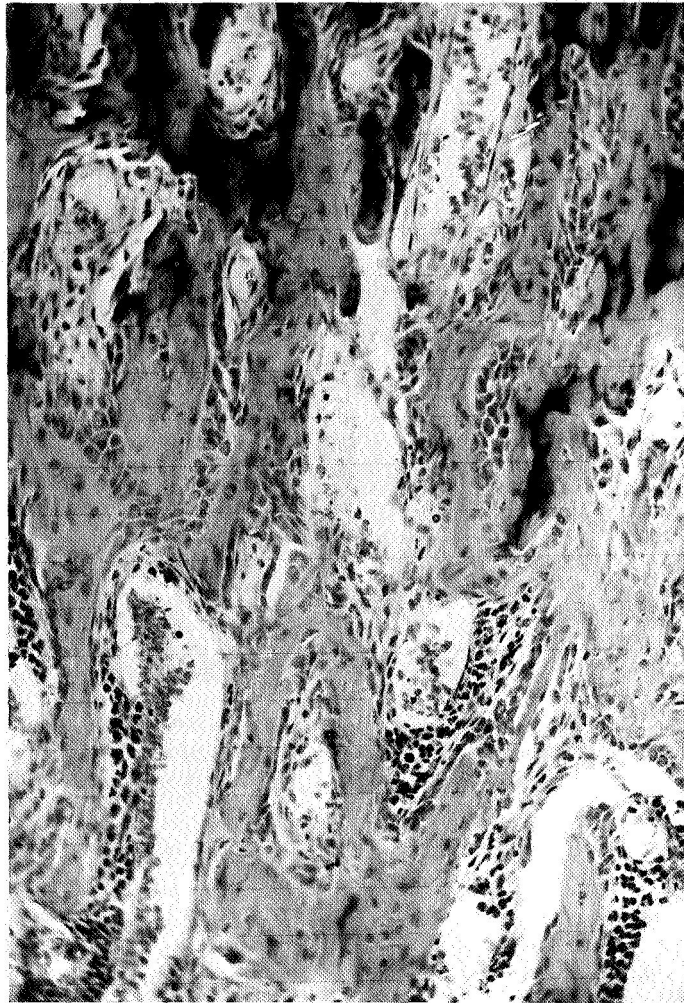


FIGURE 112. Higher magnification of figure 111 showing construction of new bone with active osteoblasts. 200 $\times$ .

hormone affects osteoblasts by suppressing their ability to synthesize protein, and reorients them to something, either back to mesenchyme cells or—well, they just change.

I think this is a matter of the sensitivity of these cells, in a particular animal species, to the dose level for this particular function of this particular hormone.

MCLEAN: This is what I was trying to emphasize, that what we are seeing in the rat is something that we have never been able to produce in any other laboratory animal. The rat has its peculiar response,



and we have never seen this hyperostosis, particularly the osteoblastic reaction with the formation of a lot of new bone in the metaphysis, in any other species.

TALMAGE: I feel that all the work that Dr. Nichols does on the effect of parathyroid hormone on inhibition of protein synthesis is an example of this type of effect. In the rat, parathyroid hormone will inhibit a mechanism in osteoblasts, probably biochemically the same type of mechanism which exists in the mesenchyme cells. However, it takes a higher dose to produce such effects in osteoblasts.

PECK: Occasionally in the clinical syndrome of primary hyperthyroidism, we do see patients who have increased bone production locally. It is not really a hyperostosis, but local increases in bone production within the confines of the bone, presenting an osteosclerosis.

MCLEAN: That is why this condition was long ago termed "osteitis fibrosa"—because in chronic cases of hyperparathyroidism a fibrous reaction with overproduction of bone and fibrous tissue has been reported.

PECK: It is bone, primarily.

MCLEAN: It is bone, but surrounding the bone there are a lot of cells that look like fibroblasts. I would like to demonstrate again the reversal that occurs spontaneously in the bird (ref. 175). Figure 113 shows sections of bones from a pigeon. Figure 113(a) is the osteoblastic stage when the pigeon is forming medullary bone, and one sees almost a pure culture of osteoblasts with every sign of a great deal of activity. Suddenly, when the egg starts moving down the oviduct and acquiring a shell, the whole picture changes and the spicule of bone (fig. 113(b)), instead of being surrounded by osteoblasts, is surrounded by osteoclasts and is undergoing rapid resorption. This lasts, in the pigeon, for about 24 hours, and during the time the egg is being coated with the shell.

We are certain that this is not a parathyroid phenomenon. We tried to reproduce this picture with parathyroid extract in birds and did not succeed. But something triggers a reversal so that in place of the osteoblasts there are only osteoclasts, and resorption occurs at a rapid rate.

NICHOLS: May I say something more about this biphasic parathyroid effect? Dr. Flanagan and I published a paper not long ago in which we tried to relate the changes in bone-cell metabolism to the amount of hormone that was being poured out, or if you like, the frequency with which the total amount of hormone was excessive (ref. 152). Some patients showed an inhibition of proline incorporation into collagen, while others showed a considerable stimulation. This seemed to be related to how active the disease was.

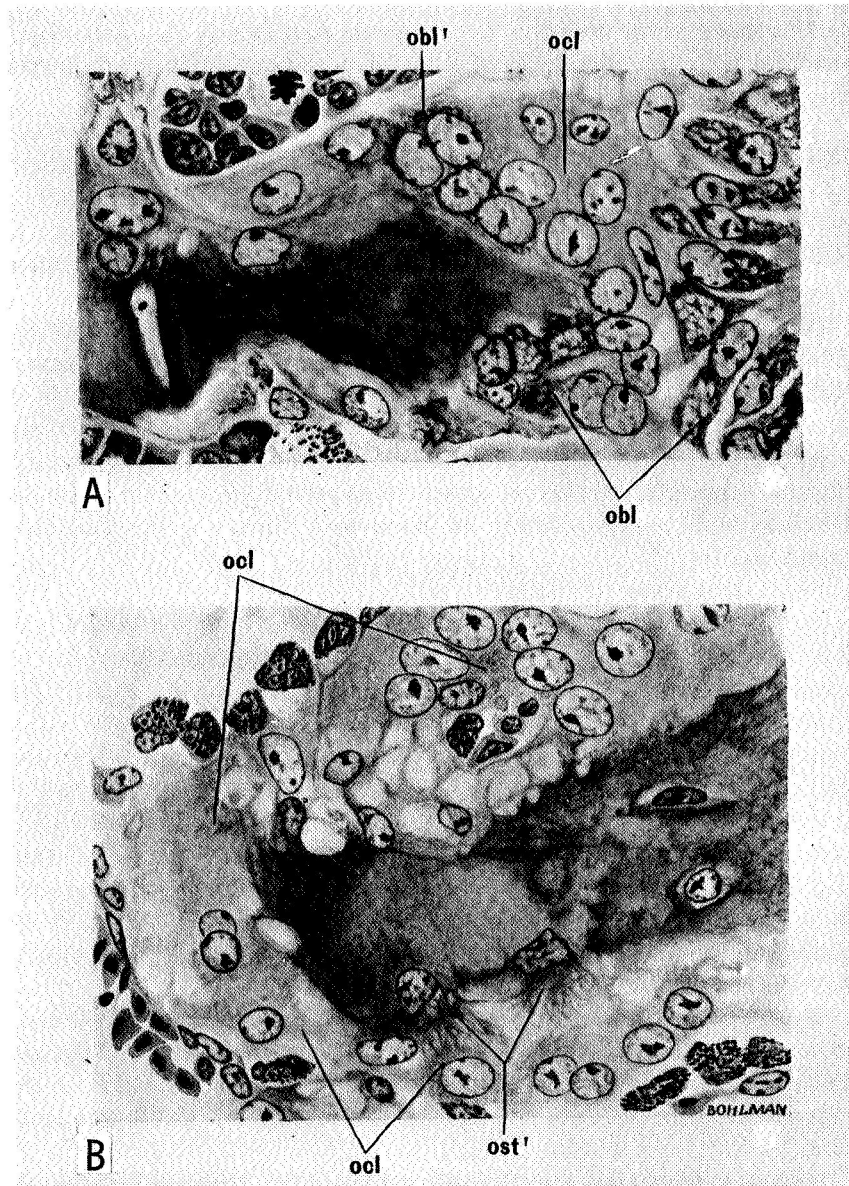


FIGURE 113. Photomicrographs of a section of bone marrow of a pigeon killed 25 hours after laying the first egg. (a) A stage in the transformation of an osteoclast (ocl) into osteoblasts (obl) through the accumulation of basophilic cytoplasm (obl') around the nuclei of the osteoclasts before the separation into individual cells. (b) During the process of breakdown of bone, liberated osteocytes (ost') fuse with an osteoclast (ocl). Zenker-formol fixation; HEA stain. 425 $\times$ . [From ref. 175; reprinted by permission of the publisher.]

Those who had flagrant disease all showed stimulation of new bone formation. We suggested that these were the patients in whom the destructive process was tremendously accelerated, and we thought we were seeing the end result of the well-known link between resorption and accretion, which seems to be present in most animal species. On the other hand, for the patients in whom there was inhibition of collagen synthesis, the diagnosis seemed to be exceedingly difficult to make. Hypercalcemia and hypercalciuria were only occasionally present and evidence of bone disease was totally lacking. Nevertheless, these people had adenomas and were apparently cured by their surgical removal.

ARNAUD: I think that we are dealing with a terribly complex situation when we try to interpret the manifestations of parathyroid hormone excess. The responses of the intact animal to the administration of parathyroid hormone are almost certainly not related exclusively to parathyroid-hormone action. There is little question that the hypercalcemia produced alters the production and secretion of other hormones as well as changing the distribution of anions and cations across biologic membranes. Thyrocalcitonin is in this regard a very important hormone.

Now, the mode of action of thyrocalcitonin is unknown, but I think we are all agreed that it acts on bone. In the studies that have been shown, we have not been told that the animals were thyroidectomized. If they were not, we must assume that they all had high circulating levels of thyrocalcitonin.

Also, much of the work that has been done *in vitro* has been with commercial parathyroid extract. It should be noted that very likely there are contaminating materials in this extract that have a profound influence on biologic systems. Any time an effect has been demonstrated *in vitro* with parathyroid extract, the same effect should be expected when the highly purified hormone is used. If it is not demonstrated, the possibility must be considered that a contaminant is causing the effect.

YOUNG: Some of us have used parathyroid extract for purposes other than elucidation of the effects of endogenous parathyroid hormone in man. We are well aware that commercially available beef parathyroid extract contains an appreciable amount of nonhormone contamination. The point of using it, and the point of using the rats, which respond very overtly to the extract, was to demonstrate that the cells of bone are capable of assuming different specializations under different environmental conditions. As I saw it, this was the significance of the work I presented here. The observation that the bone cell can assume different functional states to meet different circum-

stances can be demonstrated in many ways and, in fact, was supported several years ago by Dr. Bloom and Dr. McLean, using different systems, including the egg-laying birds which we saw here (refs. 135, 175, and 176).

It can be shown in a fracture site, or *in vitro* by changing the culture conditions, or in the induction experiments of Dr. Urist, and so on. I would just like to emphasize that I do not think there is any disagreement between Dr. McLean and myself on the ability of these bone cells to change their specialization (as some of you might have been led to believe). The demonstrated ability of the bone cell to adapt itself to changed conditions is perhaps one of the more important findings to grow out of this work.

LLOYD: Just for the record, perhaps I ought to say that at the 1967 British Bone & Tooth Society meeting on calcitonin, Dr. Gaillard presented some work on embryonic mouse radius explants. He spoke on the subject of growth and found an increase in the number of osteoclasts using parathyroid extract. By adding thyrocalcitonin, he was able to negate this effect and produce a much larger number of osteoblasts.

NICHOLS: I think Dr. Arnaud's point needs to be reemphasized, which indeed your report does, because if one removes various glands from rats one can find a whole hatful of different changes in bone-cell metabolism. For example, a pituitary must be present for normal metabolic activity; the same kind of change one obtains with hypophysectomy can be reproduced by removing the thyroid; administration of TSH to a hypophysectomized animal restores collagen biosynthesis nearly to normal. Therefore, we must seriously consider using more or less completely ablated animals if we are going to define more precisely the various primary effects of these hormones on the system.

COPP: The point Dr. Arnaud is making is that matters are complicated when a hormone is given which produces changes in ionic concentrations of calcium and phosphate in blood. This may affect the release of other hormones or may have a direct effect on bone and kidney.

ARNAUD: I do not think that we should be such purists that we exclude all of the actions, the actually observed actions, of parathyroid hormone in nonablated animals, but I do think we ought to hold some things in reserve. I wonder about the osteosclerotic type of response that is seen in the intact animal after parathyroid hormone has been injected. I think this may be related to other hormones.

TALMAGE: Just a word in opposition to this. Such a response can be produced without ever raising the plasma calcium concentration. In our experimental procedures we were able to get what I once glibly

called a "flower garden of osteoblasts" without ever pushing the plasma calcium concentration above 10 mg/100 ml.

ARNAUD: I am not talking about the classic osteoclastic response. I am a believer.

TALMAGE: I am very happy to hear you say this because for 10 years I have insisted that the best way to study parathyroid function is not to use exogenous hormone, but to cause the animal to increase its own hormone production.

ARNAUD: I think that this may be the best way of going about it. On the other hand, it is not the easiest way, and we are not all going to sit down and do peritoneal lavage.

RAISZ: There is one further proviso with regard to tissue-culture studies. Most tissue cultures employ serum in the medium, and serum is a very complex material. We have attempted to eliminate serum in order to eliminate factors that complicate our studies as well as experiments in the living animal. The hormones can be removed by using serum from thyroparathyroidectomized animals. Such serum will support bone resorption in tissue culture. When you try to get rid of the serum, the parathyroid response to bone resorption disappears. Adding back various components of serum has indicated that it is the albumin fraction of serum which supports the parathyroid response in tissue culture (ref. 177, work done in collaboration with Dr. Paula H. Stern, Department of Pharmacology, University of Michigan School of Medicine). Whether or not the factor or factors involved have any importance *in vivo* certainly deserves further study.

FREMONT-SMITH: I think we have to be extremely careful at all times, but nobody suggested that tissue culture was the answer to all problems. It has been suggested that it is the answer to no problems, and I think you have brought out very nicely the fact that you made a discovery by working with tissue culture of something which you did not know was there.

One of our problems is that we are constantly trying to take the animal apart to see how the parts work, and every time we do this we find it is much more complicated than we thought it was and we try to put it back together again. This is a constant interaction process. It is interesting that when you get a group of people together from different disciplines, each one rejects the data from the other disciplines because it is much too complicated and awfully messy. So I am just bringing out the fact that up until the time I mentioned tissue culture it had not been mentioned, and it seems to me it is a very important thing to mention with all of the dilemmas it is going to produce. It will produce another whole series of dilemmas, and they may throw more light on how large our area of ignorance is.

TALMAGE: I have been instructed by Dr. Bauer to apologize for speaking so strongly against tissue culture in the last session. I really have great faith in tissue-culture techniques, and I apologize.

URIST: This seems like the time for Dr. Copp to make some concluding remarks, and the time to extract some concluding statements from the discussants of the subject of the first session, "Homeostasis of Calcium."

NICHOLS: While Dr. Copp is preparing this material may I just tell Dr. Arnaud that as far as the sclerotic reaction described or the stimulation by parathyroid hormone is concerned, it is indeed true that one must have both a pituitary and/or a thyroid. Probably it is the thyroid, because TSH-treated, hypophysectomized animals do seem to have some response.

ARNAUD: Thank you.

COPP: In drawing the discussion of calcium homeostasis to a close, I would like to speak briefly of evidence for possible parathyroid involvement in control of hypercalcemia and then discuss some studies on dietary factors in homeostasis.

In most species, the parathyroids are embedded in thyroid or are so closely associated with it that it is difficult to separate them. In many of the earlier experiments, parathyroidectomy was performed by removing the thyroid as well, although this is not always mentioned in the paper. Now that the importance of the thyroid in calcium homeostasis is recognized, it is important to make a clear distinction between the parathyroidectomized and thyroparathyroidectomized state. In the dog, it is impossible to perfuse the parathyroids independently of the thyroid, and for this reason, our original experiments on gland perfusion did not distinguish between a thyroid or parathyroid origin for calcitonin. However, in the sheep and pig, the superior parathyroid which makes up 90 to 95 percent of the total parathyroid tissue, is quite distinct with a separate blood supply, so that the two glands can be perfused independently (ref. 178). As shown in figure 114, high calcium perfusion of the thyroid in sheep caused little change in plasma calcium concentration at 1 hour, but there was a slow and steady fall thereafter. The response to high calcium perfusion of the parathyroid was a prompt fall in systemic plasma calcium. This observation has been confirmed by Care et al. (ref. 179), who also observed a hypocalcemic response to injections of certain parathyroid extracts. MacIntyre et al. (ref. 180) observed, in dogs, that the fall in plasma calcium concentration was greater when thyroid and parathyroids were perfused with high calcium blood than when only the thyroid was involved. However, the evidence that parathyroid calcitonin may play a role in controlling hypercalcemia in some species is still tenuous,

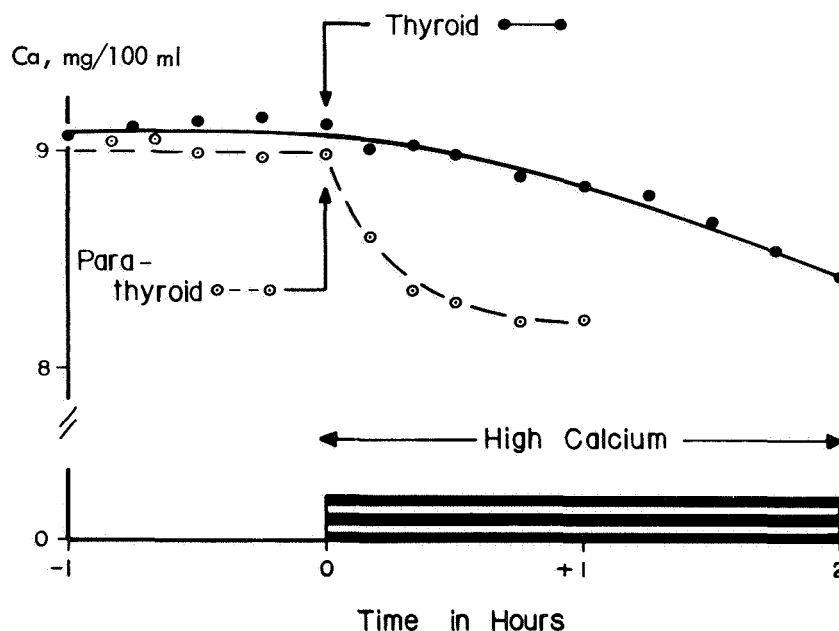


FIGURE 114. Changes in plasma calcium associated with high calcium perfusion of the thyroid or the superior parathyroid in 18 anesthetized sheep.

particularly when compared with the very substantial evidence for an important function for thyroid calcitonin.

I would now like to turn to our studies on the effect of dietary calcium and phosphate on calcium homeostasis in young rats. These experiments were carried out by Miss Anne Kuczerpa, who has developed an excellent ultramicro method for calcium, based on color development with glyoxal bis(2-hydroxyanil). Each determination requires only 0.02-milliliter plasma, so that as many as 10 samples of tail blood may be analyzed in the same animal.

The composition of the synthetic diet is given in table XXIII. It uses purified beef fibrin as the protein source and is essentially free from calcium and phosphate unless supplemented as indicated. It also contains approximately seven times the minimum requirement of vitamin D.

One of the real problems with a synthetic diet is to persuade the rats to eat and, as shown in figure 115, we go to considerable lengths to make it attractive. The most important feature is the rum flavor.

With the low phosphate diet, there is a fall in plasma phosphate and a rise in plasma calcium concentrations within 24 hours. This is apparent even when calcium is also absent from the diet, as shown in

TABLE XXIII

COMPOSITION OF THE CALCIUM- AND PHOSPHATE-FREE DIET (PER KG)

Basic ingredients:		Vitamins— <i>Continued</i>	
Beef blood fibrin.....	g 350	Folic acid.....	10
Corn starch.....	380	Niacin.....	50
Sucrose.....	175	Ca pantothenate.....	50
Alphacel.....	40	Inositol.....	200
Vitamin A oil.....	2		g
Vitamin D oil.....	2	Ascorbic acid.....	1.0
Vitamin mix.....	25	Choline.....	1.8
Salt mix (varies).....	17		IU
Salts:		Vitamin A.....	20 000
Sodium bicarbonate.....	g 14.6	Vitamin D.....	8 000
Potassium carbonate.....	1.39	Supplements: g:	
Ferric citrate.....	.5	A. Normal Ca (0.40%) Low P	
Magnesium chloride.....	4.2	(0.02%):	
Potassium chloride.....	.95	Add:	
	mg	Calcium carbon-	
Cupric sulfate.....	75	ate.....	10
Manganous chloride.....	143	Sodium bicarbon-	
Potassium iodide.....	.22	ate.....	14.6
Cobaltous chloride.....	5	B. Normal P (0.40%) Low Ca	
Zinc chloride.....	2	(0.01%):	
Vitamins:		Add:	
Thiamine HCl.....	10	Sodium bicarbon-	
Riboflavin.....	10	ate.....	14.6
Pyridoxine HCl.....	5	NaH <sub>2</sub> PO <sub>4</sub> · H <sub>2</sub> O.....	8.5
PABA.....	5	NaHPO <sub>4</sub> .....	8.5

figure 116, although the effect is not so great. This would suggest that it is the low phosphate concentration in blood rather than the calcium in the gut that is responsible. There is some indication that the low concentration of blood phosphate inhibits bone formation and increases osteolysis, thus raising the calcium concentration even when none is available from the gut (ref. 181). The effect of the phosphate-deficient diet was also observed in thyroparathyroidectomized rats. As shown in figure 116, within 24 hours of feeding a low-calcium, low-phosphate diet, the plasma calcium concentration had returned to normal, and within the next 2 days, there was a slight hypercalcemia. Certainly, it is rather surprising to obtain hypercalcemia in a young parathyroidectomized rat which has been fed a calcium-deficient diet. It also indicates that phosphate may have an important role in calcium homeostasis, even in the absence of the thyroid and parathyroid glands. We found that the presence or absence of the thyroid made no difference in the degree of hypercalcemia in these animals, and this agrees with our





FIGURE 115. Typical procedure for feeding rats the synthetic diet.

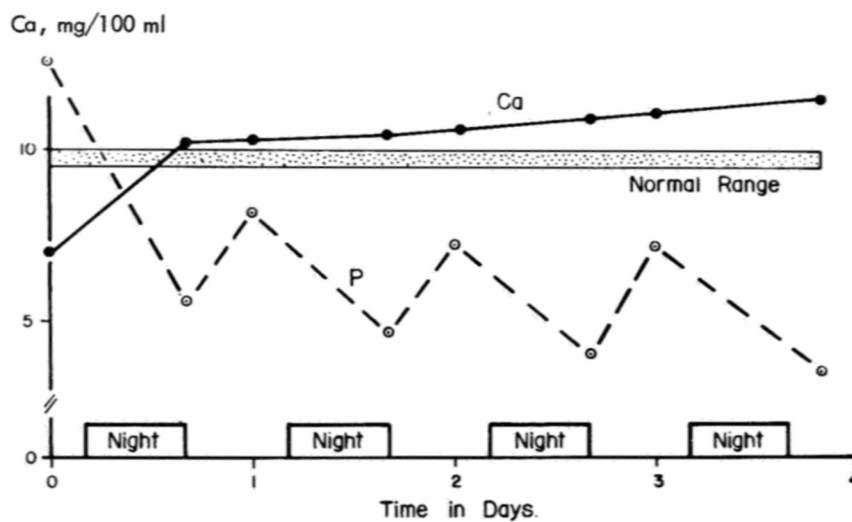


FIGURE 116. Changes in plasma calcium and phosphate when 5-week-old rats that had been thyroparathyroidectomized 24 hours earlier were fed a diet low in calcium (0.05 percent) and phosphorus (0.02 percent). Note the diurnal fluctuations in plasma phosphate. The points represent an average for seven rats.

observation that the response to both parathyroid hormone and thyrocalcitonin is greatly reduced in the rats which have been fed a phosphate-free diet. I should point out that these rats were not truly deficient because they had been fed the diet only for a few days at the most; thus,

the effects are more probably a result of the fall in blood phosphate concentration, which occurs almost immediately.

Feeding these month-old rats a diet with normal calcium content but free from phosphate resulted in hypercalcemia and hypophosphatemia within 24 hours. Even more interesting was the apparent effect on parathyroid function after 3 days on such a diet. The gland appeared to be nonfunctional; as shown in figure 117, the very slow fall in plasma calcium concentration that occurred during the day when the rats were fasting was essentially the same following parathyroidectomy as it was after sham operation. The similarity is even more striking (fig. 118) when hypocalcemia is produced in these animals by intraperitoneal injection of phosphate. The curves for the sham-operated and parathyroidectomized groups are the same, indicating essentially no homeostatic response to hypocalcemia.

TALMAGE: Have these rats been maintained on a low phosphate diet for only 3 days?

COPP: Yes, and I do not think there was time for any real deficiency changes in bone. In my opinion, the significant factor is the fall in plasma phosphate concentration.

We studied the effects of varying the levels of both calcium and phosphate in the diet on the response to parathyroid hormone and thyrocalcitonin, and the results are shown on table XXIV. The re-

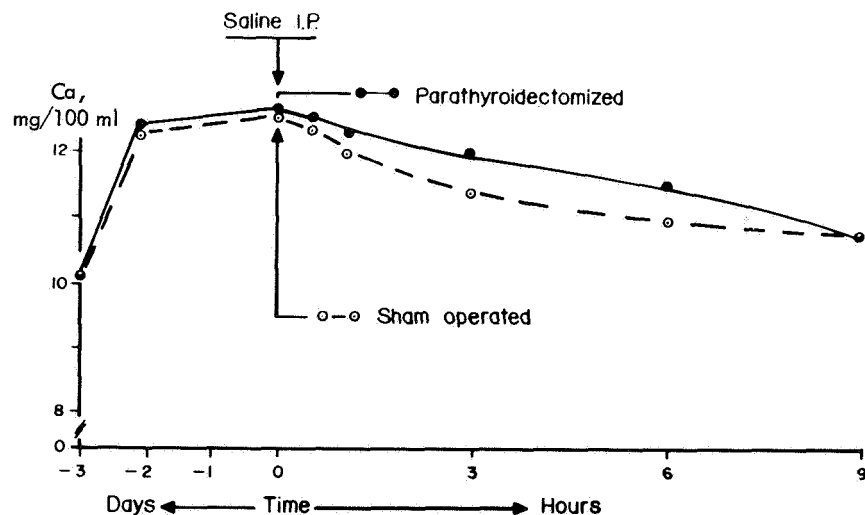


FIGURE 117. Changes in plasma calcium concentration in 5-week-old rats during the 3 days on the low phosphate diet and after parathyroidectomy or sham operation. The fall in calcium during the daytime fast is normal on this diet and is associated with an increase in plasma phosphate concentration. Note that removal of the parathyroids has essentially no effect.

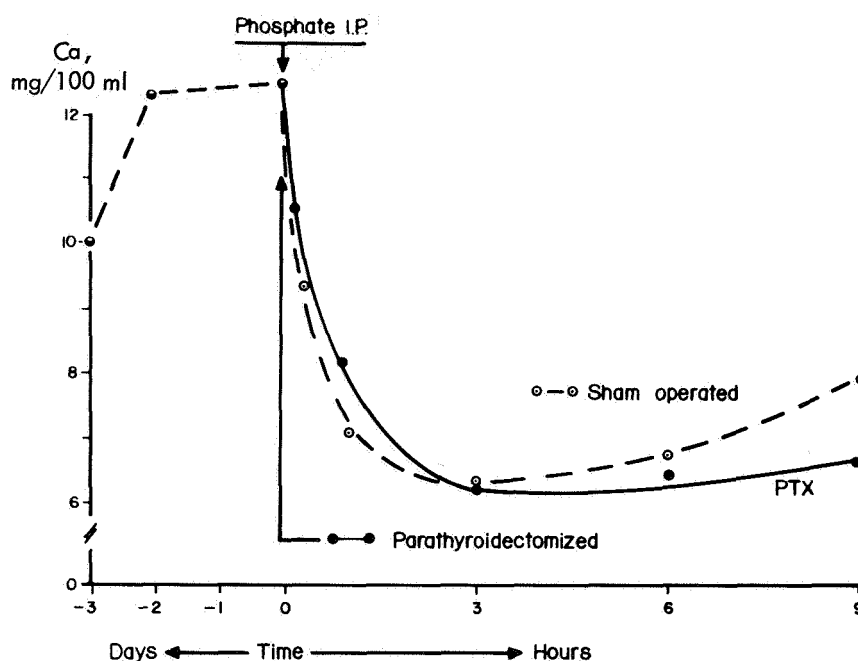


FIGURE 118. Changes in plasma calcium concentration in 5-week-old rats during the 3 days on the low phosphate diet and after intraperitoneal injection of inorganic phosphate and parathyroidectomy or sham operation. Note that there is essentially no homeostatic response for at least 6 hours, and the sham-operated animals behave in the same way as the parathyroidectomized group.

sponse to hormone was significantly reduced on the phosphate-free diet. This table gives a very reliable indication of response to the hormone and correlates very well with the logarithm of the dose administered in the case of either parathyroid hormone or thyrocalcitonin.

TABLE XXIV

EFFECT OF DIETARY CA AND P ON THE RESPONSE TO PTH AND TCT

	A	B	A	B	A	B
Dietary Ca, percent:						
0.80.....	3.1	2.10	6.5	9.5	17.5	10.7
0.40.....	7.4	2.30	18.0	9.6	9.2	10.6
0.01.....	1.6	2.8	11.1	16.7	8.5	15.2
Dietary P, percent.....	0.02		0.40		0.80	

NOTE.—Numbers represent area response between curve for plasma calcium concentration (mg/100 ml) against time (hours) after injection of parathyroid hormone (PTH) or thyrocalcitonin (TCT).

Col. A: area response to 1U of PTH/g; col. B: area response to 2 Hirsch U of TCT/g.

It will be seen that the responses to both hormones are significantly reduced in animals which are fed the phosphate-free diet. The best response to parathyroid hormone was obtained when there was adequate calcium and phosphate in the diet and the calcium-to-phosphate ratio was 1 to 1. The best thyrocalcitonin response was obtained with a calcium-free diet and adequate phosphate.

In conclusion I would like to refer again to figure 3 which showed the main factors involved in calcium homeostasis, including the enormous reservoir of calcium in the skeleton, and the two calciostats which control the plasma concentration in the normal animal: first, the parathyroid, releasing parathyroid hormone in response to hypocalcemia, and second, the thyroid, releasing thyrocalcitonin in response to hypercalcemia. The manner in which they may act is shown in figure 119. In this young dog, when the plasma calcium concentration was lowered by infusion of EDTA, the calcium rose rapidly after the infusion, presumably as a result of the action of endogenous parathyroid hormone released by the stimulus of hypocalcemia. However, the rapid increase stopped abruptly when the normal range was reached. This was presumably a result of the fast action of thyrocalcitonin,

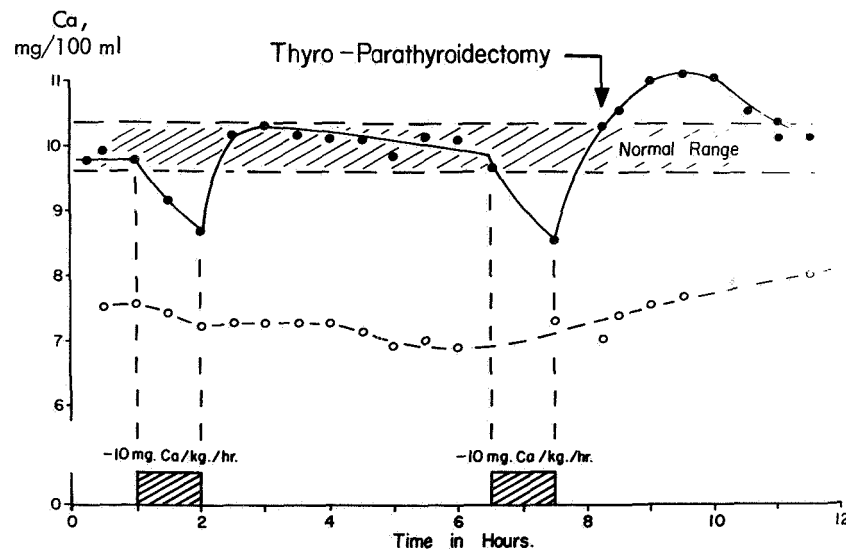


FIGURE 119. Evidence for the homeostatic role of the thyroid and parathyroid glands showing the recovery from EDTA-induced hypocalcemia with glands intact and in the same dog when thyroparathyroidectomy was performed (arrow). Note the overshoot in plasma calcium concentration, indicating loss of the very precise control of hypercalcemia when the glands are present. [Adapted from ref. 42; reprinted by permission of the publisher.]

because this sharp cutoff did not occur when the experiment was repeated and the thyroid and parathyroids were removed.

BAUER: May I register one protest? I think it is strange to show a scheme of this type and leave out vitamin D completely. There is considerable evidence that vitamin D has something to do with calcium metabolism.

COPP: I agree completely. Vitamin D is certainly a critical factor in calcium metabolism and I am sure that deficiency of this essential vitamin will interfere with normal calcium homeostasis. However, because of the limited time available, I hesitated to open this Pandora's box. Vitamin D could occupy a full session at some future time. We will put into the record that vitamin D has an important role in calcium metabolism.

BAUER: Two generations of children have been brought up on it.

URIST: We must stop at this point and go on with Dr. Owen's presentation.

OWEN: You have heard something about the various types of bone cells in a population. We have been looking at the pattern of RNA synthesis *in vivo* in the different cells of bone using autoradiographic techniques. The system we study is the actively growing periosteal surface of the midshaft of the femur of 1-week-old rabbits. The actively growing periosteal surface has characteristic loops representing forming primary osteons (fig. 120(a)). In figure 120(b) the loops close up to form canals in the process of bone growth, and each contains a blood vessel and some osteogenic cells. The osteoblasts, which form a single layer of cells on the bone surface, have the characteristic appearance of very active osteoblasts. Behind them is a thicker layer of cells which we have called preosteoblasts; these cells are the precursors of the osteoblasts. Behind these is a layer of fibroblasts about three cells thick which separates the osteogenic tissue from muscle. The osteocytes are embedded in the bone matrix. Within the haversian canals, cells which are on the bone surface have been called osteoblasts; cells within but not on the surface have been included in the category of preosteoblasts (fig. 120(a)).

In the previous work we have measured various parameters of this system and it was found that in these young actively growing animals the preosteoblasts and osteoblasts spend on the average of 3 days on the bone surface before becoming enclosed in bone, either as cells within the haversian canals or as osteocytes embedded in the bone matrix. The bone surface advances, in the process of bone growth, at the rate of about 70 microns per day, as illustrated in figure 120(b). It was thus possible to calculate the rate of production of matrix, and it was found that each osteoblast produces approximately its own volume of organic bone matrix per day during the 3-day period it spends

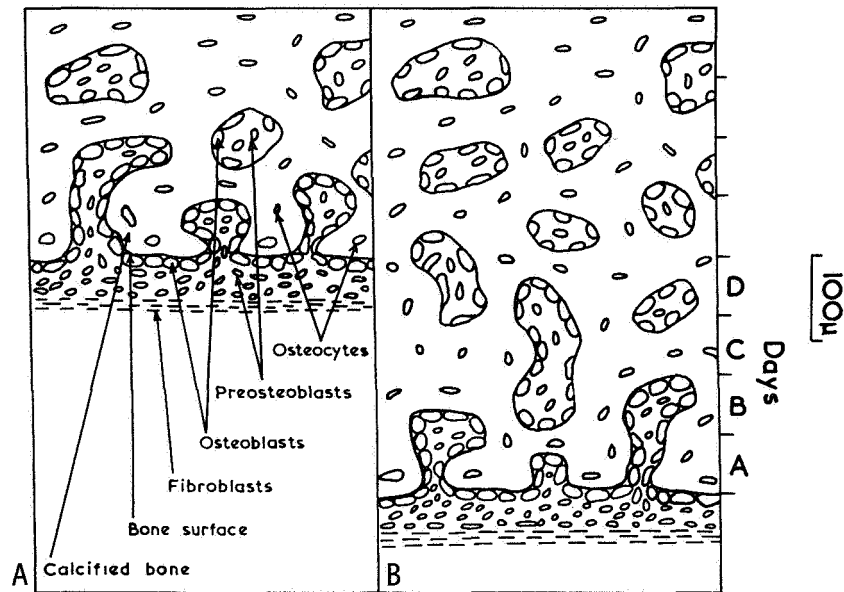


FIGURE 120. (a) Diagrammatic representation of part of the periosteal surface of a 1-week-old rabbit, the femur shaft. (b) The position after 4 days' growth.

on the periosteal bone surface. In addition, the endosteal bone surface in this region is lined with osteoclasts which are resorbing bone from this surface at approximately the same rate at which it is being laid down on the periosteal surface. A study of these osteoclasts was also made.

Here, then, is a system that includes different stages of cellular differentiation. First, the preosteoblasts engaged mainly in cell proliferation. Second, the osteoblasts on the bone surface, which are in a state of maximum functional activity, engaged in the synthesis of a few specific substances and in particular the protein, collagen. Third, the osteocytes and "haversian osteoblasts" which are a later and more quiescent state of the cell. Finally, the osteoclast which is responsible for bone resorption. It seemed of interest to investigate RNA synthesis and turnover in this system, particularly in relation to its pattern in the various cell types, representing, as they do, different stages of differentiation of the same cell.

Rabbits, 1 week old, were given a single intraperitoneal injection of  $^3\text{H}$ -uridine as the RNA precursor and killed at different times after injection. The bones were fixed in 10 percent neutral formalin, decalcified, and embedded in paraffin. Sections, 5 microns thick, were cut

and autoradiographs prepared using the stripping-film technique. Counts were made of the number of grains per nucleus and per cytoplasm in the different cell types. The results up to 10 days after injection for the preosteoblasts and osteoblasts on the periosteal surface are shown in figure 121. It should be noted that the same pattern of labeling is followed both for preosteoblasts and osteoblasts; that is, there is an initial rapid appearance of label in the nucleus with a slower appearance of label in the cytoplasm. This is similar to the results obtained so far for other cell types. The curves for the nuclear labeling are almost identical for the preosteoblasts and osteoblasts (fig. 121); in the case of the cytoplasm the curves have a similar shape, but the level of labeling in the cytoplasm is lower in the preosteoblasts. The level of labeling in the cytoplasm does not reach its maximum until at least 18 hours after injection and it then falls off very slowly. The osteoblasts are most active during the 3 days which they spend on the bone surface, and are in fact producing, on the average, their own volume of protein per day during this period. An estimate of the rate

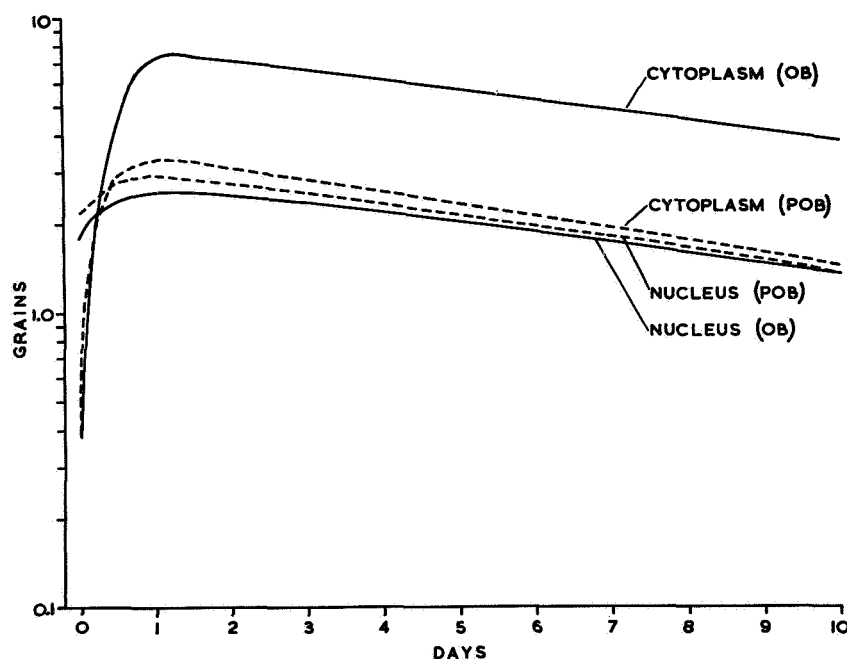


FIGURE 121. Grains per nucleus and per cytoplasm plotted against time after a single intraperitoneal injection of  $^3\text{H}$ -uridine. The experimental points have not been included. The curves for nuclear labeling in the two cell types are almost identical. In the case of the cytoplasm, labeling is slower than in the nucleus and reaches a higher value in the osteoblast than in the preosteoblast.

of loss of RNA during this period can be determined from the falloff in the retention of labeled RNA between 1 and 4 days after injection. As can be seen from figure 121, the rate of loss of RNA during this time is about 8 percent per day.

Our results for the osteoclasts are not so complete. However, what results we have show that they follow a very similar pattern to those of the other two cell types. It is also of interest that we have found a difference in uptake in the osteoclasts in different regions of the bone, presumably depending on differences in osteoclastic activity.

PRITCHARD: Uptake of what?

OWEN: Tritiated uridine. Now I would like to mention the results for the osteocytes. We find two types of labeling of osteocytes. At short times after injection we see a low level of labeling in the osteocytes which are close to the growing bone surface. This can be explained in terms of a low rate of protein synthesis which continues in the most newly formed osteocytes after they have been embedded (ref. 112).

However, the second type of labeling, which will now be described, is of particular interest, since it illustrates an effect of environment on the functional apparatus of the cell. The fate of an osteoblast after its period of maximum activity on the bone surface is, in the process of bone growth, to become either an osteocyte embedded in bone matrix or an osteoblast on the surface of haversian canal (fig. 120(b)). Cells at any position *A* or *B*, for example, will be of the same age, and may be either osteocytes or "haversian osteoblasts." They will be radioactively labeled by virtue of the fact that they have taken up the radioactive precursor while they were osteoblasts on the surface at the time of injection. Figure 122 shows a comparison of the amount of RNA label remaining in these two types of cells of the same vintage, at three different positions—*A*, *B*, and *C*—in animals killed 3 or 4 days after injection. You will remember that position *A* was nearest the bone surface and these are the most recently formed osteocytes and "haversian osteoblasts." Positions *B* and *C* are deeper within the bone, respectively. The graph shows the ratio of the grains per osteocyte to the grains per osteoblast for the different positions. At position *A* the osteocyte has approximately the same grain count as the osteoblast, showing that it becomes embedded containing approximately the amount of RNA that it had as an osteoblast. At later times, i.e., at positions *B* and *C*, the osteocyte has lost its RNA, whereas the osteoblast retains its label. The implication of this result is that the osteocyte finding itself in a situation where it presumably is not going to need its protein synthesizing apparatus for some time is instructed under the effect of its new environment to abandon it. This result can be seen visually on a histologic section where the osteocytes near the growing



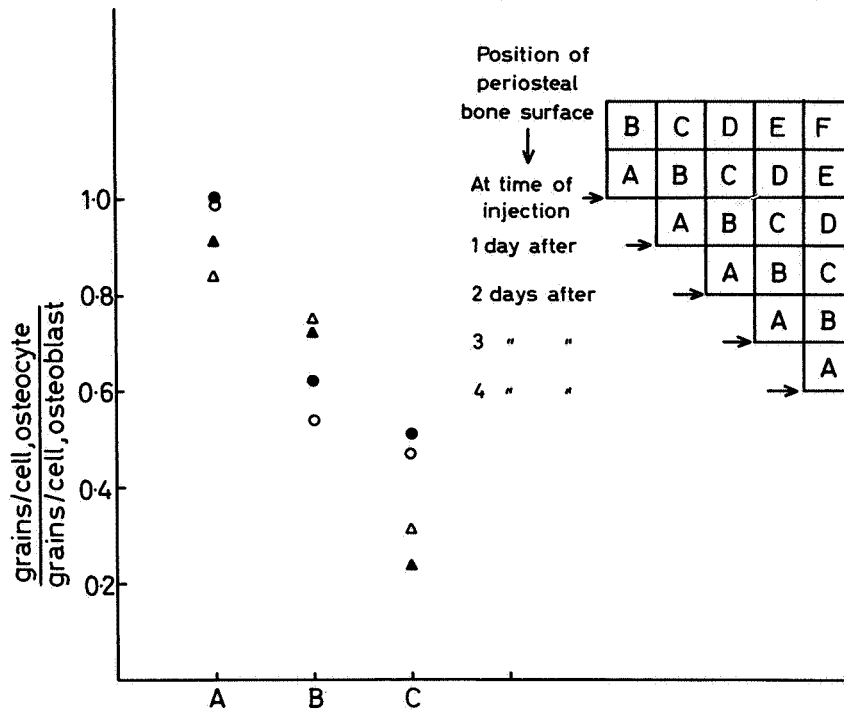


FIGURE 122. The ratio of grains per osteocyte to grains per osteoblast at different positions with reference to the bone surface. (See fig. 120.) The results are for four animals: three were killed at 3 days and one at 4 days after a single intraperitoneal injection of  $^3\text{H}$ -uridine.

bone surface have cytoplasm full of RNA while there is a gradual loss of cytoplasm in osteocytes at increasing distance from the bone surface.

Finally, I would like to refer briefly to some other experiments that are relevant to the interpretation of the above results. The fact that one does not know how much of the label has been removed by the various histologic procedures is a limitation of the autoradiographic technique as used with paraffin sections. Recently we have made considerable progress in determining this, using the technique of frozen-section autoradiography which has not previously been applied to bone. Using this technique, it is possible to obtain autoradiographs of frozen sections in which the soluble components are retained. The technique is briefly as follows. Pieces of film were placed on a coverslip with the emulsion side up (fig. 123). These were cooled to  $-20^\circ\text{C}$ , the temperature of the cryostat. The bones were frozen in liquid nitrogen at  $-180^\circ\text{C}$ . Frozen sections were cut on the cryostat and picked up on the surface of the emulsion; this had to be done in the darkroom. This combination was then put away for autoradiographic exposure. After exposure the section was fixed and the film developed and the whole

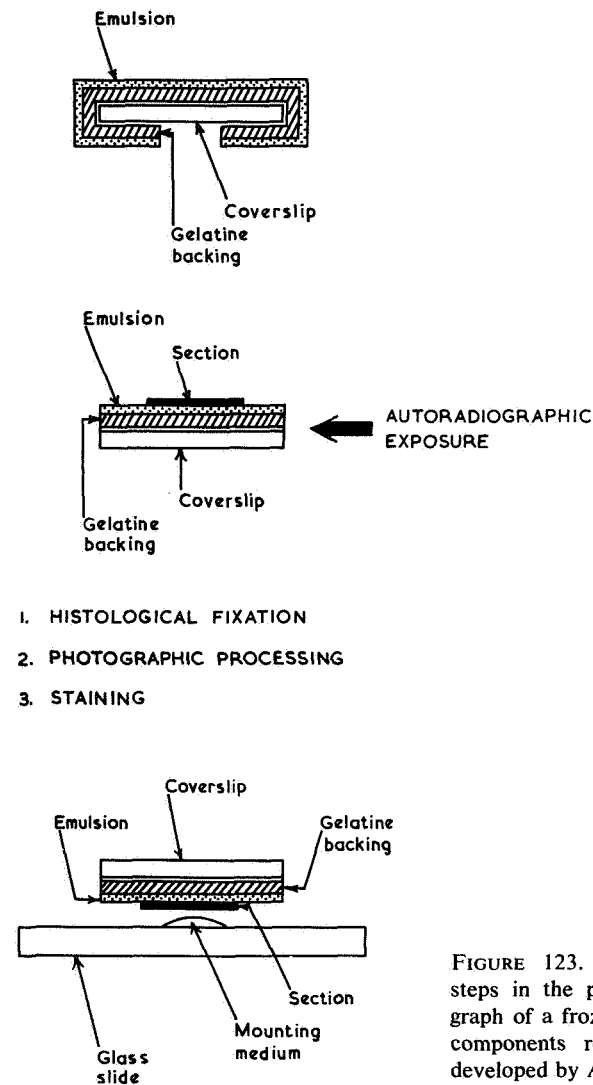


FIGURE 123. Diagram of the different steps in the production of an autoradiograph of a frozen section with the soluble components retained, using the method developed by Appleton (ref. 182).

combination inverted onto a slide for observation. In the process of fixation and development, of course, water-soluble components will be removed, but this does not matter since their image on the film has already been obtained (ref. 182).

It is possible to extract a frozen section to remove certain components and to compare the autoradiograph of the extracted section with that of a frozen section that has not been extracted. For this purpose we have extracted sections using the fixative, formol-acetic-alcohol solution, and compared these with autoradiographs of frozen sections where

all the components are retained. The radioactively labeled insoluble material remaining is considered to be representative of macromolecular RNA. The results show that at 1 hour after injection, only 14 percent of the label present is in RNA, the other 86 percent is washed out by the acid fixative. By 7 hours, about 50 percent is in RNA and by 24 hours it appears that a small fraction may still be extractable, although it is necessary to repeat the process to confirm this. It was also noted that more extractable material is washed out of the cytoplasm than out of the nucleus.

BÉLANGER: This is still uridine?

OWEN: Yes; we were using radioactive uridine in these experiments.

BÉLANGER: Do you interpret that as free uridine?

OWEN: It is probably labeled and has been incorporated into the RNA precursor pools, which contain small or intermediate-sized molecules.

BÉLANGER: It would not be just the label that is there?

OWEN: Yes; there could be some free uridine—I do not know. In time after injection, less of the label is in the extractable form and more is in the nonextractable form or macromolecular RNA. We are going to repeat and extend these experiments over the whole time scale.

I would like very briefly to give a quick résumé of the main points that I have made, which are relevant to the present discussion. The pattern of RNA synthesis and decay in osteoblasts and their precursors, the preosteoblasts, and also in the osteoclasts is qualitatively similar. The amount of RNA appearing in the cytoplasm, however, is greater in the osteoblast than in the preosteoblast. An interesting effect of environment on the retention of RNA was observed in the case of the osteocyte. Up to 24 hours after injection a significant fraction of the radioactive label is in an acid-extractable form.

RAISZ: I wanted to ask about the very slow loss of uridine from cells in your culture. I wonder if this represents reutilization of the label, since the decay rate for RNA for most tissues appears to be faster than this. The half times for uridine look to be 10 to 15 days in your material.

OWEN: Yes.

RAISZ: If this is true, this might explain that at 24 hours, the free material which you find actually represents a breakdown of reutilized uridine nucleotides or other soluble precursors.

OWEN: Yes; the half-life of RNA as measured from our curves is between 7 and 8 days. This is not so different from other measurements which have been made *in vivo*; for example, Loeb et al. (ref. 183) have found a value of about 5 days for the half-life of ribosomal RNA in rat liver. However, I agree with you that there is likely to be reutilization of RNA breakdown products and this is one of the problems in

interpreting RNA studies. I think further experiments using frozen-section autoradiography may give us some idea of how much of this occurs.

RAISZ: If this is so, then the decrease you see in the labeling of the osteocytes may be a function of stopping RNA synthesis and not of jettisoning the RNA of the cell. If reutilization has been maintaining the slow slope in the osteoblasts, the steeper slope in the osteocyte could simply represent decrease in synthesis rather than accelerated breakdown or actual secretion of RNA.

PRITCHARD: You have another bit of evidence. The osteocytes are smaller bodied, less basophilic, which is some indication of the total amount of RNA.

RAISZ: The question is whether it gets smaller by less synthesis or by getting rid of something.

PECK: I would like to compliment Dr. Owen on an elegant presentation. I think this is really fascinating work.

I have one question about the technique of washing. Binding of small amounts of tracer to macromolecules without actual incorporation is always a problem for the biochemist, and very often almost impossible to eliminate. I was wondering if you had tried, for example, adding stable uridine to the washing fluid to see whether this would make any difference.

OWEN: No; that would be worth doing, but I have not tried that. I have tried flushing out with stable uridine after injection of the labeled uridine. If I give an injection of stable uridine, hundredfold, 2 hours after injection of labeled uridine, it has no effect on my results.

URIST: I would like to turn the floor over to Dr. Currey for some concluding remarks to his earlier presentation.

CURREY: I am afraid I am going into the realm of the "wild-blue-yonder" theory—no elegant experiments, or anything like that. I want to talk briefly about the other aspects of local factors involved in remodeling, that is, how bone as a whole adapts itself by growth or remodeling, external remodeling, responding to unbalanced stresses that may fall on it; why femurs end up straight from one end to the other—that sort of question.

My thoughts on this were stimulated by Frost's book (ref. 184), which some of you may have read. He is rather overheavy on the mathematics of things, I think, but he certainly has some interesting ideas.

One has to consider under what circumstances bone needs to change its external form. I am going to use as an example an extremely simple system. I am only going to consider a long bone, without considering much about muscle pulls or the like—merely a long bone that should end up more or less straight.

If you have a long bone that is habitually (or more often than not) subjected to a simple bending stress, there is no particular alteration of its external form, except by increasing the total amount of bone tissue, that will make it any better at resisting that stress. In all cases, by increasing the total amount of bone tissue, you can, of course, reduce your liability to fracture, but one cannot go on increasing weight indefinitely, and presumably the safety factor cannot reach more than a certain level.

Similarly, if you have a bone which is loaded in pure compression, or in pure tension, there is nothing special that it can do to increase its resistance to fracture.

But when you get slightly more complicated systems, then there is something that the bone can do. For instance, suppose that the bone is loaded habitually in compression, and also is bent at the same time. If this bone is loaded habitually, more by being bent to the right rather than to the left (fig. 124(a)), then what the bone can do to reduce its liability to fracture is to become, when unloaded, bent to the left (fig. 124(b)). In other words, it should grow from being straight, into a bow. This perhaps may explain the slight forward bowing of the femur, for instance, because when it is loaded in compression it tends to bow forward even farther, and then the bending force of the adductors will tend to pull it back in, in the opposite direction, so the bending stress that would be produced by the adductors alone will be changed

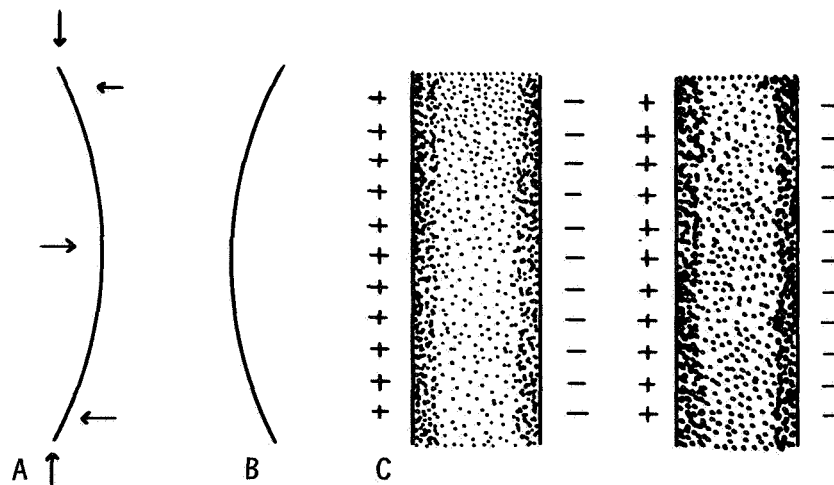


FIGURE 124. Long bone subjected to stress. (a) The bending produced by forces acting on an originally straight bone. (b) The shape of bone after adaptive growth that would counteract the bending forces. (c) Vertical section through the middle of the shaft, showing where bone must be added (+++) and where bone must be taken away (---).

into some slight increase in the compressive stress, and bones, of course, are good at resisting compression and not very good at resisting bending.

If you consider the shaft—the vertical section in figure 124(c)—to bring this adaptive bowing about, you have to resorb bone on the surfaces indicated by (–) and add bone on the surfaces indicated by (+).

Similarly, if you have a bone that is bent initially (fig. 125(a)) and is subjected to pure compression, the bowing itself is going to produce a bending moment; therefore, you want to straighten up (fig. 125(b)), and to do this, bone must be resorbed and added to.

Now, for such a relatively simple system, all that the bone—the accretion-resorption mechanism—needs to know at any particular point is that stress has occurred, and the change in the curvature of the surfaces.

FREMONT-SMITH: Stress has occurred, or stress is occurring?

CURREY: Both. One assumes it is going on the whole time. One has to integrate this over a certain time and, of course, living systems are very good at integration.

You also have to make a change in the curvature; and for the bone to appreciate that stress has occurred, it must be extremely simple. There must be hundreds of ways of doing this. On the other hand, to measure the change in curvature of a surface is very difficult indeed, particularly with the slight amount of strain that you will get in something like bone; but Frost (ref. 184) suggested that this is what is measured. The amount of change in curvature will be very small, but the change in strain through the thickness of the shaft can be measured. For example,

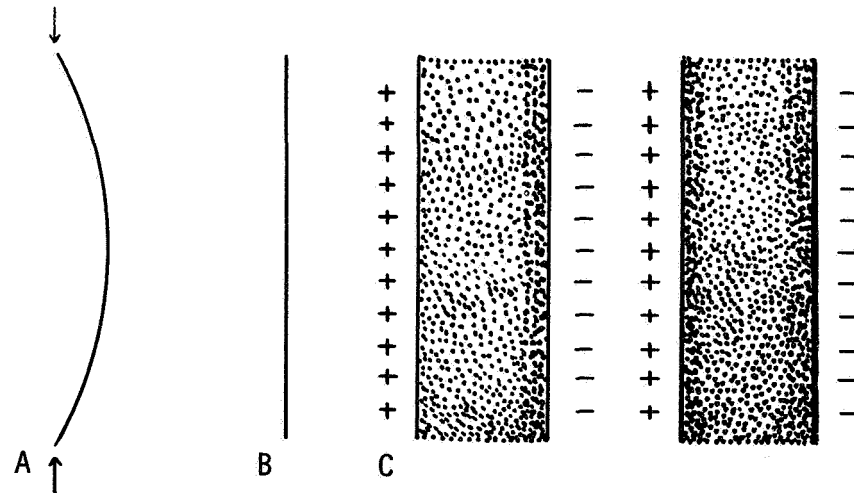


FIGURE 125. Same as figure 124, except that the bone is originally bowed to the right and is subjected only to longitudinal compression.

take the case shown in figure 124(a), where the bone is loaded axially and bent. Now let us look at the section through the cortex shown in figure 126(a). The left periosteal surface in this figure is going to be loaded in compression because of the axial stress, and additionally in compression because it is being bent out to the right, so we can represent the stress by three C's on the left-top figure. Then the left endosteal surface is a bit nearer the neutral axis so that there will be a lower stress, represented on the right by two C's.

The right endosteal surface is loaded in tension because of the bending, but there is also a longitudinal compression component. If you add these two—let us say for present purposes they add up to nothing—there is actually no stress at that endosteal surface, and the right periosteal surface is in tension because, although there is longitudinal compression, the bending more than counteracts it.

If you draw a horizontal line above which there is compression and below which there is tension, then you have high compression on the left, and tension on the right (see fig. 126(b)). In other words, there is a change in the compression or tension as you go in deep from a surface. Therefore, if you can measure the change in the state of

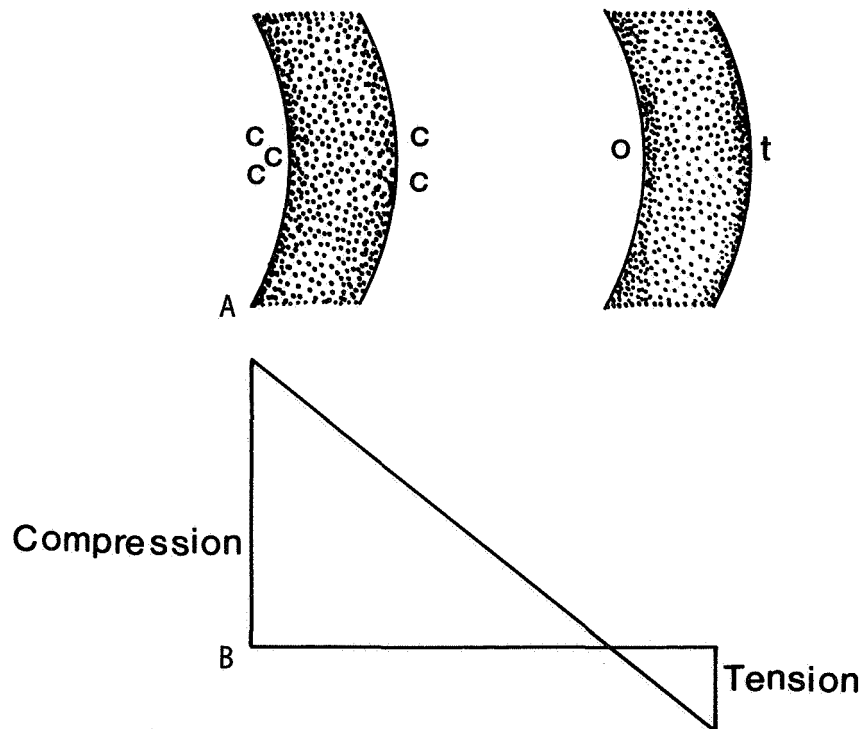


FIGURE 126. The state of stress in a longitudinal section of a bent bone.

stress as you go into the bone from a surface, you can, in effect, measure the change of curvature that has occurred at that surface. And with the system that I have talked about so far, you could devise a simple rule, and that is: Take away bone from a surface if the strain gets more compressive as you go in, and vice versa.

This is Frost's system (ref. 184). The modification I have introduced is not measuring surface curvature which is probably impossible, but measuring changes in stress as you go in deep. This works for both endosteal and periosteal surfaces. Bassett (ref. 185) produced a simple scheme which enabled his electric phenomena to explain changes in adaptive growth, and if you followed his system and worked out what happened, you would find that you would get one side of the bone disappearing entirely and the other side increasing in width; this would not be a satisfactory arrangement. You must have a system that can tell the endosteal surface to behave in a different way from the periosteal surface.

How do we measure this change in stress? I do not know, but Bassett and Becker (ref. 186), and others, have shown that electric potential generated by the stress would seem a very simple way of measuring stress. The canaliculi in bone, of course, making connection with each other in depth seem to be absolutely tailor-made for measuring changes in stress, or maybe ionic fluxes over some little distance of the order perhaps of even a millimeter, which would be quite sufficient.

The system of Frost (ref. 184), with the modification that I have introduced, would work perfectly well with the rather simplified system I have used if bone were never loaded in tension as a whole. The bone was loaded in compression axially, but because of bending, the right side was loaded in tension and the left side might have been very much in compression, but if you add up all of the stresses of a particular section and divide by the number of places you measured, you find it comes to compression.

If you ever have a net tensile stress on a long bone, then this system of Frost's will produce the wrong answer. If, for example, you have a bent bone loaded in tension, you get the wrong answer; you get an opposing feedback system, with more and more bend until the bone breaks. So this will not work if bones are loaded in tension.

PRITCHARD: There are one or two other things. You have left out the periosteum, which is under tension and which could itself be a receptor.

CURREY: Yes, true.

PRITCHARD: And you could get a feedback from the periosteum.

CURREY: Yes.



PRITCHARD: Another factor again is reflex contraction which will tend to reduce tension and replace it by compression. Bone is not just a piece of wood.

CURREY: I am not thinking for a moment that it is. Could I take those points separately? We will take the second one first and say that I quite agree that muscles, of course, will reduce unbalanced stresses a lot, but there will probably be residual unbalanced stresses, particularly if the bone is the wrong shape.

With reference to the periosteum, I think the periosteum is very important. However, what the periosteum cannot do on its own is measure a change of stress with depth. I will bring in the periosteum directly.

If we have to consider loading in tension, what you find is that with this simple rule I have suggested about taking bone away, you get more compression; if this works, if the bone is loaded in net compression, and if the bone is loaded with net tension, then you have simply to reverse the rule.

When I first came across this, I thought, "Now we can just work out a way in which you can reverse the rule." All you have to do for the section as a whole is to know whether it is loaded under net tension or net compression. This is extremely difficult, because what it means is that any particular part of the bone has to get information in some way about the stress acting on the bone as a whole. One cannot do it by any simple method of measuring the flux at a particular point of measurement. You have to get information which comes from other parts of the bone, and possibly this is where the periosteum will come into play.

The minimum information required, in more or less symmetric circular bone, would be, at any particular point on the bone, to have information about the stress there and the stress diametrically opposite. If you just add the stress at these two points and take the mean, as it were, this will tell you whether there is net compression or tension.

But it seems to me somewhat unlikely that you have rather neat nerves going around to diametrically opposite parts of the bone, and what I would like to inquire of you who know something about histology of bone is whether anything in detail is known about the arrangement of the nerve fibers in the periosteum, and to some extent in the endosteum.

All I have shown here is the minimum amount of information that is required for a system to change the shape of a bone in an adaptive fashion. It sounds quite small. We just want to know the change of stress at depth, and the net stress on the whole tension. In fact, I think the practical difficulties are going to be rather large.

ROBINSON: The point is brought up that if you are going to try to convert mechanical energy into cell activity, you need some sort of

receptor system, and there is a system of nerves in the body which is, I think, often overlooked. The system of C-fibers is a very positive system (Sir Thomas Lewis (ref. 187), discussed this in his book on pain), because pain does arise from the deep somatic structures, and we are constantly faced with the presence of this system. We do not know exactly how it works, but nevertheless it is there.

Dr. Milgram, a student who was working with us on the cortex of the femur of dogs, trying to cut haversian canals, and Dr. Cooper, similarly employed, suddenly ran into the presence of nerves in haversian canals (refs. 25 and 188). This has been described before with certain histologic techniques, but here we have definite morphologic evidence that such nerves are present in the haversian canals of dogs.

The adult animal has a paucity of osteoblasts in the haversian region. However, in the area such as the one shown in figure 127, between the osteoblast and the endothelial cell, one sees a Schwann cell. This is more than occasional. I should say they are found in more than half of the canals in the adult animal in the middle of the femur; that is, halfway between the periosteum and the endosteum of the cortex.

Figure 128 is an excellent electron micrograph by Dr. Cooper and is of higher magnification than figure 127. It shows part of two endothelial cells of the capillary in this haversian canal; the basement membrane is clearly seen. The adjacent Schwann cell has its own basement membrane. The small C-fibers are invaginated into the Schwann cell's plasma membrane. One can see bundles of collagen fibrils in this region around the Schwann cell. Although the Schwann cell lies between an endothelial cell and an osteoblast, we have never observed the nerve fibers going to endothelial cells or osteoblasts in haversian canals of the femur of the adult dog. We have never observed these nerves at all in the young growing puppy.

FREMONT-SMITH: Have you done any nerve degeneration studies?

ROBINSON: Yes, Dr. Milgram is now working on this.

FREMONT-SMITH: This ought to give a very sharp clue, ought it not?

ROBINSON: We hope that it will. Of course, we feel that it has implications in, for instance, Paget's disease, where it appears that the cells are not behaving in any orderly, controlled fashion in response to stresses and strains.

BÉLANGER: May I ask Dr. Robinson to elaborate on how the nerves would influence Paget's disease? Is that a vascular change that you expect, or what? Are these autonomic nerves?

ROBINSON: This is pure speculation.

BÉLANGER: Are they autonomic nerves, do you think?

ROBINSON: This is the type of nerve fiber which is associated with deep somatic innervation. The same type of fiber is seen in the various ligaments; for instance, the anterior and posterior longitudinal ligament

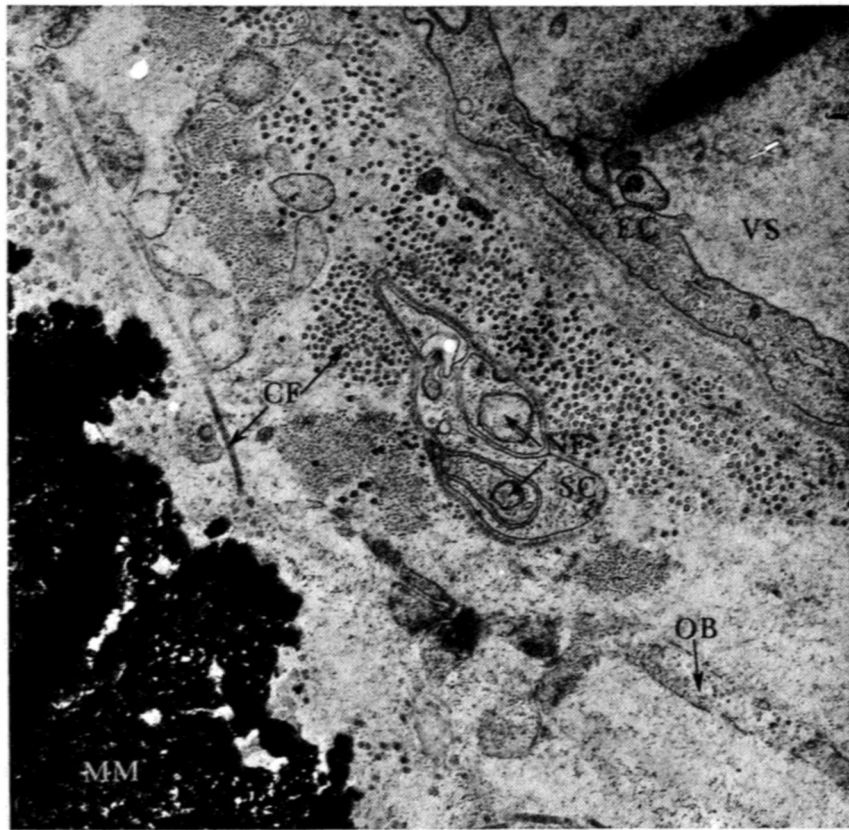


FIGURE 127. Electron micrograph of the space between the endothelial cell (EC) wall of the haversian vessel and the mineralized bone matrix (MM) at the periphery of a completed haversian canal from the femoral cortex of a dog. In the upper right corner is the vascular space (VS). Pinocytic vacuoles are seen in the endothelial cell wall. A junction between the endothelial cells is seen at the top of the picture. The separate basement membrane is observed along the avascular side of the endothelial cells and around the Schwann cell (SC). The nerve fibers (NF) are invaginated into the Schwann cell. Collagen fibrils (CF) are scattered throughout the extracellular, extravascular fluid space between the endothelial cells and the mineralized bone matrix. The osteoblast (OB) cell cover of the bone matrix seems fenestrated and incomplete.

In this preparation, the collagen fibrils stained more densely than the cement substance, unlike those in figure 14. Moreover, critical inspection of the mineral deposits on the periphery of the mineralized bone matrix suggests that the primary mineralization here may be in the collagen fibrils rather than between them, as in figure 14. Eventually, both the fibrils and space between are obliterated by mineral crystal density (left lower corner). Approximately 23 600 $\times$ .



FIGURE 128. Electron micrograph of a section from the femoral cortex of a dog showing, in detail, parts of two endothelial cells (*EC*) and a Schwann cell (*SC*) with eight nerve fibers (*N*) invaginated simply into its plasma membrane. In the specialized regions, i.e., between the plasma membrane of two endothelial cells, there are usually two of these desmosome-like regions between the junction of two cells in the capillaries of the haversian canals in dogs. Osteoblast (*OB*) atrophied, or resting, in this instance; red blood cell (*RBC*) in lumen of haversian capillary; intermediate cell (*IC*). Approximately 27 200 $\times$ .

of the spine, the annulus of the disk. This type also accompanies the small vessels which permeate our whole system. Perhaps I should point out the difference between this nerve and the myelinated nerve. Would that be helpful?

BÉLANGER: Yes.

ROBINSON: It has been clearly demonstrated by Robertson (ref. 189) that a myelinated or A-nerve fiber varies not only in size, having gen-

erally a much greater diameter than C-fibers, but also in the way it is invaginated into the Schwann cell's plasma membrane; it is wrapped in a spiral invagination of the plasma membrane of the Schwann cell. Since the plasma membrane is a lipoprotein, its multiple layering about the A-fibers forms the myelin cover of those nerves. Thus, by size and by the type of invagination into the Schwann cell one can morphologically distinguish the myelinated A-fibers from the smaller unmyelinated fibers of which the C-system is composed.

URIST: Thank you. We will have another presentation on cell physiology, and as soon as we get some cell physiology into this information-and-control concept, we will return to the subject of cellular differentiation. I am going to ask Dr. Howell to proceed.

HOWELL: For the past 6 years we have been studying processes involved in calcification of epiphyseal cartilage in normal and rachitic calves as well as in normal and rachitic rats.

The part of our recent work which I thought might be of interest has to do with a new system we have developed for studying the epiphyseal plate cartilage with respect to a fluid phase which we have been able to aspirate from the cartilage.

URIST: By micropuncture?

HOWELL: Yes. Earlier in the conference, regulation of mineral-phase transformation was discussed by Dr. Nichols and Dr. Bélanger, and views were advanced that certain key events might mediate osteoblastic regulation of mineral deposition in bone, including, among other possibilities, active transport of mineral ions into the mineralizing site, removal of water from the site, increase of pH, or formation of nucleation centers. To obtain direct evidence for such regulator factors and to evaluate their relative importance—if multiple factors are involved—is now virtually impossible in bone. However, for a similar biologic system, we have for the first time been able to sample and study a fluid phase; i.e., in hypertrophic cell cartilage of rachitic rats before and during calcification *in vivo*. I would like to present briefly some data on this preparation because they offer potentials for directly testing mechanisms of mineral-phase separation as influenced, for example, by parathyroid hormone, thyrocalcitonin, or vitamin D.

Our experimental preparation is illustrated in figure 129. A normal or rachitic rat under Nembutal anesthesia is placed on a plastic operating table similar to that used for renal micropuncture studies. The limb is fixed in 45° flexion and bathed with oil at 37° C. A finely adjustable vacuum pump is used to control an oil column which is communicated to the surface of the cartilage through the micropipet. As you know, in renal micropuncture, micropipets with tips of 5 to 10 microns are placed on a manipulator and guided under direct visual-

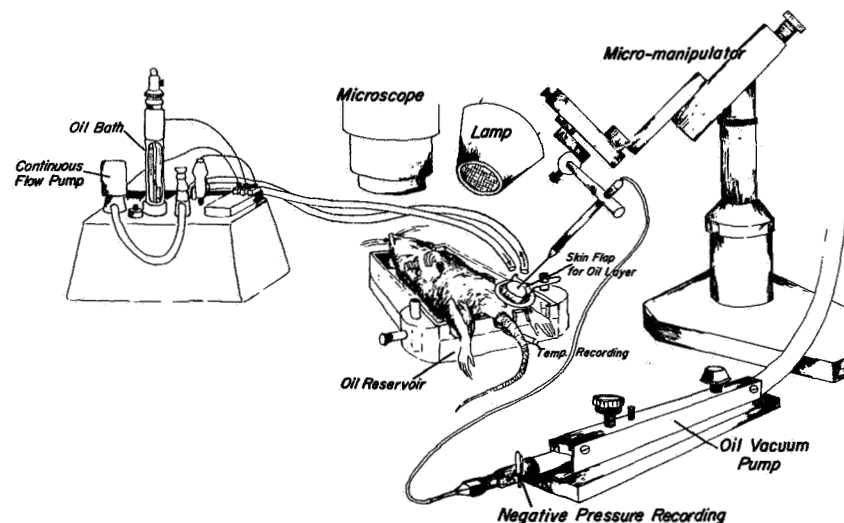


FIGURE 129. Assembly for use of cartilage micropuncture technique. [From ref. 190; reprinted by permission of the publisher.]

ization into renal tubules; fluid is aspirated and samples studied with respect to various parameters of renal tubular transport.

Here we employed the same approach to obtain fluid from the cartilage plate. We aspirate in exactly the same manner. The operative stress of this procedure is less than that resulting from renal micropuncture. Only a nick is made medial to the extensor tendon, and our open tissue exposure is small (ref. 190).

FREMONT-SMITH: Where do you get the fluid? Do you get the fluid from that device?

HOWELL: We have evidence that the micropipet tips are placed between the rows of hypertrophic cells of the cartilage plate. Fluid that is obtained from such a site is clear and shows no fibers or cells when observed under a phase microscope. The volume is from 20 to 40 millimicroliters, collected during 10 or 15 minutes at a negative pressure sufficient to overcome tip resistance—300 millimeters Hg.

This fluid is obtained easily, but is of such small volume that we had to devise ultramicro methods for analysis of calcium, phosphate, protein, and nucleotides. For this purpose, we utilized a Zeiss PMQ II spectrophotometer with a Zeiss No. 507425 special sample changer; we constructed a Bakelite positioner and an ultramicro cuvette (fig. 130) of 8.4-microliter total capacity (ref. 191). With this cuvette, direct quantitation of these parameters down to level  $10^{-10}$  grams has been achieved with a satisfactory range of recoveries (refs. 190 and 191).

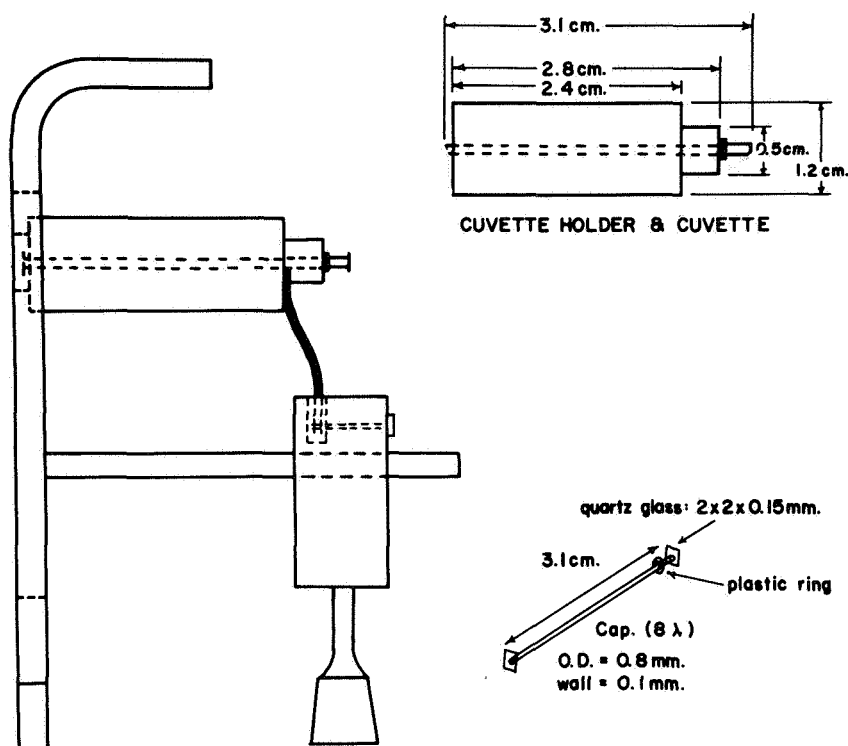


FIGURE 130. Ultramicro cuvette and positioner employed for spectrophotometric analysis of calcium and other parameters of microscopic cartilage fluid samples. [From Howell et al., ref. 191; reprinted by permission of the publisher.]

Now let us consider the question of the site of fluid collection posed by Dr. Fremont-Smith. After collection of samples, we replace a micropipet in the cartilage with the manipulator in the same position and inject a ferric chloride marker contained in Carbowax. Histologic sections are made from the collection sites and these are subjected to Perl's reaction for iron (fig. 131). The exact site of obtaining fluid is estimated from relevant histologic sections. Thus, we have obtained the hypertrophic cartilage cell fluid samples, designated henceforth as  $Fl_{SA}$ , less than 0.5 millimeter deep to the cut made in the surface of the cartilage plate and less than 0.2 millimeter distal to the junction between cartilage plate and bone epiphysis (fig. 132). The site of fluid collection from the resting cell cartilage,  $Fl_{SB}$ , was about 0.2 millimeter or less from the surface cut.

Next, we determine that the fluid  $Fl_{SA}$  was not derived from perichondrial surface fluid leakage. To do this, we placed a 1-percent solution of Iodocyanine Green in 0.9 percent saline on the nicked perichondrial surface of the cartilage and made routine aspirations of



FIGURE 131. Photomicrograph of rat upper tibial epiphyseal plate. Approximate site of collection of microscopic fluid samples assessed by injection through micropipet of a marker indicated at point of arrow. Phloxine stain. 120 $\times$ .

$Fl_{SA}$  from the hypertrophic cell region after varying periods up to one-half hour (fig. 133). The ultramicro cuvette readings for these aspirated samples indicated less than 4 percent contamination with the dye-containing surface fluid (ref. 190).

We also made consecutive attempts to obtain fluid from tissue sites adjacent to hypertrophic cell cartilage represented in figure 134. The percentages of attempted micropuncture aspirations that were successful in obtaining a clear fluid were: 90 for the hypertrophic cell region; 75 for the resting-cell cartilage next to the perichondrium; and 95 for the perichondrial tissue and muscle insertion sites. No fluid could be aspirated from resting-cell or articular cartilage, and only



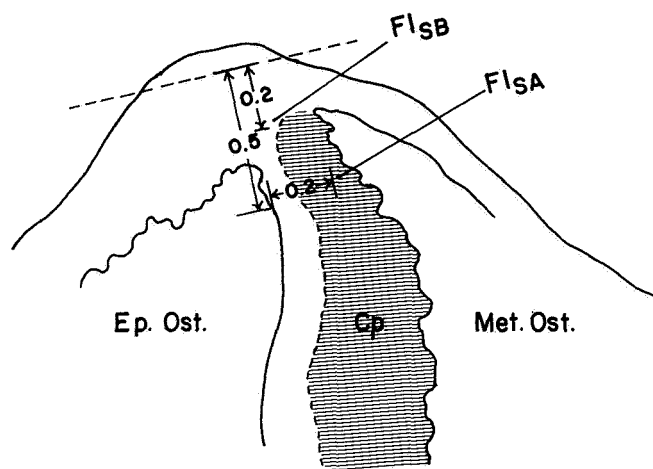


FIGURE 132. Collection site determined from a study of serial histologic sections of tissue, such as illustrated by figure 131, during the insertion of micropipet. Fl<sub>SA</sub> indicates site of hypertrophic cell cartilage fluid collection; Fl<sub>SB</sub> designates site of fluid from resting cell cartilage near surface; cartilage plate, Cp; epiphysis, Ep. Ost.; metaphysis, Met. Ost.

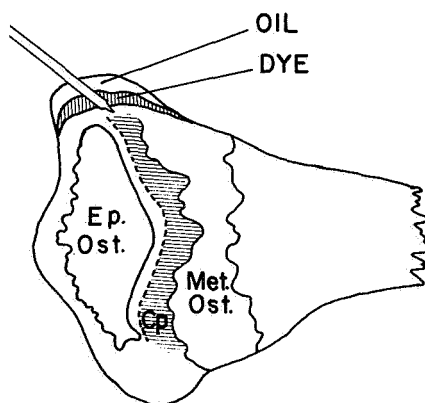


FIGURE 133. Diagram indicating position of micropipet during collection. In experiments with Iodocyanine Green, contamination of Fl<sub>SA</sub> was less than 4 percent. Cartilage plate, Cp; metaphysis, Met. Ost.; epiphysis, Ep. Ost.

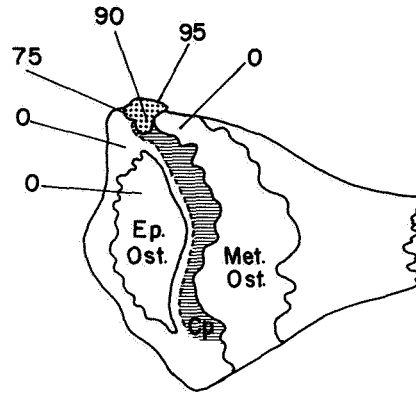


FIGURE 134. Percentage of attempted punctures, in serial trials (15–26 rats per tissue site), that yielded a satisfactory fluid sample. Cartilage plate, Cp; epiphysis, Ep. Ost.; metaphysis, Met. Ost.

blood was obtained from bony tissues. Brief mention should be made of other experiments on the fluid origin. Successful fluid collections were made from hypertrophic cell cartilage dissected free from the metaphysis and incubated in a concave slide under oil. The experiments indicated that most of the fluid was drawn from the hypertrophic cell cartilage per se and not aspirated through channels in the cartilage from a distant site (ref. 190).

Subsequent efforts were directed to ascertaining whether  $Fl_{SA}$  represented intracellular fluid, a result of cell damage, rather than a phase bathing the extracellular matrix. Chloride was quantitated in  $Fl_{SA}$  for 10- to 20-millimicroliter volumes (table XXV). Such fluid was found to be essentially extracellular due to its chloride content averaging about 100 mEq/l, corrected for protein.

FREMONT-SMITH: What is the total protein content? This could tell us whether or not it is really like plasma.

HOWELL: Total protein averaged a little over 7 g/100 ml in serum, 3 g/100 ml in  $Fl_{SA}$ , and 1 g/100 ml in  $Fl_{SB}$ .

FREMONT-SMITH: Did you ever get any fluid from normal rats?

HOWELL: Despite technical difficulties due to the narrowness of the hypertrophic cell cartilage of normal endochondral plates, we have recently succeeded in making such aspirations. In preliminary data, the protein content is about 2.5 g/100 ml. We have substantial data on the normal epiphyseal plate.

FREMONT-SMITH: The fluid in the normal is more like an edema

TABLE XXV

ANALYSIS OF MICROSCOPIC FLUID SAMPLES ( $Fl_{SA}$ ) ASPIRATED BY MICROPUNCTURE  
FROM PLATE CARTILAGE OF UNTREATED RACHITIC RATS

	Units	Number of animals	$Fl_{SA}$	Serum
Chloride.....	mEq/l.....	8	<sup>a</sup> 100 ± 2	107 ± 3
Protein.....	g/100 ml.....	15	3.1 ± 0.2	7.0 ± 0.5
Nucleotides.....	mg/100 ml.....	4	80 ± 15	18.0 ± 9
Hemoglobin.....	mg/100 ml.....	25	< 50	< 50
Calcium.....	mmoles/l.....	12	1.95 ± 0.10	2.40 ± 0.12
Pi <sub>TCA</sub> .....	mmoles/l.....	15	2.20 ± 0.2	0.65 ± 0.1

<sup>a</sup> Standard deviation; applies to tables XXV to XXVII.

fluid with very low protein, and this fluid that you are sampling is about half-diluted plasma, so far as protein is concerned.

HOWELL: Possibly. However, the fluid appears to be much more viscous than plasma manipulated in the same volume under oil and one suspects some differences in composition.

PRITCHARD: Is the hypertrophic cartilage in rickets normal?

HOWELL: No differences from normal in the utilization of pyruvate, glucose, and lactate by cartilage slices *in vitro* from similar phosphate-depleted rachitic animals has been shown in the studies of Kunin and Krane (refs. 192 and 193). Such metabolic alterations are restored toward normal in their studies by phosphate repletion with or without vitamin D. However, during such phosphate repletion *in vivo* or during incubations *in vitro*, mineral-phase separation appears discretely within the septa of hypertrophic cell cartilage, as in the normal plate.

With respect to characterizing  $Fl_{SA}$ , as listed in table XXV, the level of nucleotides is higher than that of serum, as estimated from the 260/280-millimicron ratio correlated with total protein (ref. 190). There was no detectable hemoglobin, and total calcium was approximately two-thirds of the plasma level. A striking positive finding was the concentration of the inorganic phosphate Pi<sub>TCA</sub> in  $Fl_{SA}$ , a level twice that in the serum. Our subscript for this parameter is important, since it indicates that histidine-linked or other acid-labile phosphates would register in total values for inorganic phosphate (ref. 194).

URIST: Does the high level of phosphate occur in the animal with low-phosphorus, vitamin-D-deficiency rickets?

HOWELL: Yes; the plasma phosphate concentrations indicated a severe degree of rickets. Two preparations of interest have come out of studying this fluid. We have incubated slices of cartilage prepara-

tions in calcium phosphate solutions (Yendt's solution) for 18 hours in a manner dictated by the studies of Sobel et al. (ref. 195), where they attempt to define early preformed mineral in the cartilage slices. I will not call them crystal nuclei, but some early mineral form is identified, they believe, if one incubates these cartilage slices in iodoacetate and magnesium for 18 hours. This was done in our studies, and correlations were made between the level of phosphate in the fluid and the histologic appearance of these slices. After studying about 14 different experimental preparations of the cartilage of rats treated in different ways by different dosages of vitamin D and varying phosphate intake, we found that with the administration of sodium phosphate, first done by Dr. Robinson here, I believe—

URIST: It actually was first done by Dr. McLean, on rachitic rats. He reported this experiment in which he gave solutions of phosphate to phosphorus-deficient rats, and demonstrated that he could initiate calcification in the epiphyseal cartilage without any vitamin D.

MCLEAN: It was presented before the Biochemical Society at a federation meeting (ref. 196).

URIST: My first experiment in his laboratory was to inject phosphate buffer in rats with healing fractures. We made observations on the callus of healing fractures in these phosphorus-deficient rats (ref. 197).

HOWELL: Excellent. In any event, after 2 hours of giving sodium phosphate preparation to a rachitic rat, the cartilage under electron microscopy did not show anything except staggered early dense areas suggesting mineral. After incubation for 18 hours—

URIST: Incubated in what?

HOWELL: Two percent of Yendt's solution (ref. 198), at a Ca times P product of 45 mg<sup>2</sup> percent, in the presence of magnesium and iodoacetate, showing, we believe, some alteration had occurred in the matrix as a result of having given the sodium phosphate. The interesting feature is that we have been unable to show any rise of calcium phosphate in this cartilage fluid.

Subsequently, rachitic rats were given an oral dose of 1500 units of vitamin D and  $Fl_{SA}$  samples at 0, 2, and 24 hours. During early stages of healing, inorganic phosphate increased in plasma and  $Fl_{SA}$  with no detectable alterations of the high  $Fl_{SA}$ /serum ratio (fig. 135). A similar study of the calcium concentration of  $Fl_{SA}$  in littermates treated in the same manner showed a slight and inconsistent increase (fig. 136). In this experiment all animals, including controls, had mild rickets due to a boost of serum phosphate from dietary privation 2 to 3 days previously and mineral appeared rapidly as evidenced by positive von Kóssa histologic sections of cartilage plates.

Having the total calcium and inorganic phosphate concentrations of cartilage fluids for this and a variety of other experimental rachito-

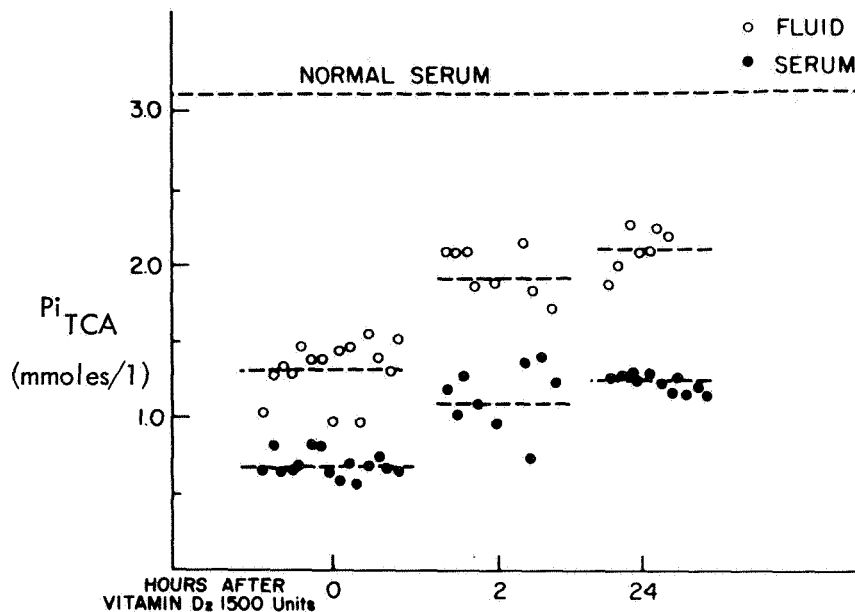


FIGURE 135. Inorganic (and acid-labile) phosphate,  $Pi_{TCA}$ , of hypertrophic cell cartilage fluid obtained by micropuncture ( $Fl_{SA}$ ) before and after treatment with vitamin D. Each open circle indicates a  $Fl_{SA}$  value for one rat, and each corresponding closed circle, serum value for the same animal. Dashed lines represent mean serum values for 10 untreated normal control rats of the same age.

genic regimens, it was relevant to assess the nonprotein-bound calcium and phosphate. As of now, we have data on animals with severe rickets (table XXVI). For this measurement, we scaled down the method of Loken et al. (ref. 199), wherein serum-protein-bound calcium is assessed following ultracentrifugation. A special holder (fig. 137) was fitted to the Model L Spinco ultracentrifuge. Micropipets containing 20 millimicroliters of original cartilage fluid under  $CO_2$ -equilibrated oil were prepared with a bubble containing nitrogen and 5 percent  $CO_2$  in the tip, sealed, mounted in the holder, and centrifuged at 105 000 g for 12 hours at 12° C. Following centrifugation, the sediment, together with the tip, was cut off under the dissecting microscope and supernatants analyzed for calcium and phosphate. Alternate samples were analyzed for pH, using an ultramicro pH glass electrode assembly with measurements under  $CO_2$ -equilibrated oil (ref. 190).

Rat serum centrifuged in this manner with a 20-millimicroliter starting sample provided a level of about 60 percent nonprotein-bound calcium, whereas the cartilage fluid ( $Fl_{SA}$ ) level was about 65 percent, at the pH levels listed in table XXVII. Protein concentrations in the

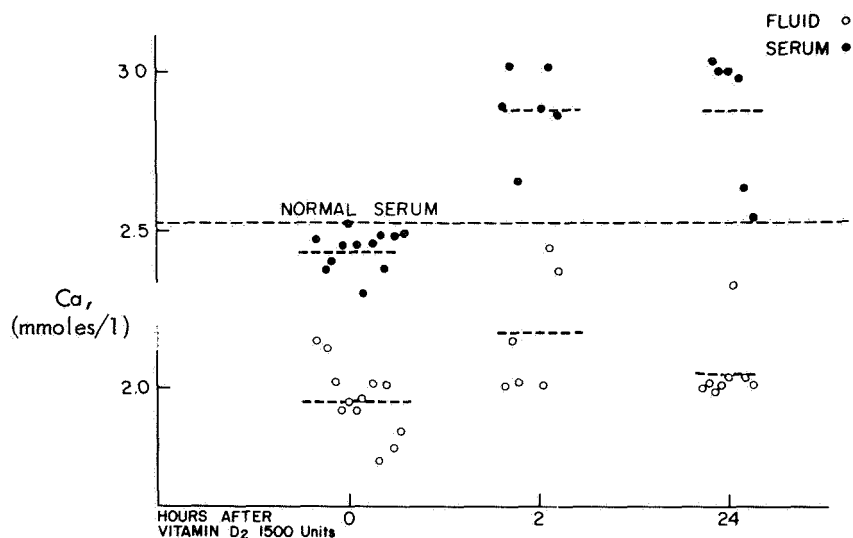


FIGURE 136. Total calcium concentration for serum and  $Fl_{SA}$  of littermates of rats in same experiments as figure 135. Each open circle indicates a  $Fl_{SA}$  value for one rat, and each corresponding closed circle, serum value for the same animal. Dashed lines represent mean serum values for 10 untreated normal control rats of the same age.

TABLE XXVI

TOTAL CALCIUM, NONPROTEIN-BOUND CALCIUM, PROTEIN, AND pH OF RACHITIC RAT SERUM AND CARTILAGE FLUID ( $Fl_{SA}$ )<sup>a</sup>

	Serum (20 $\mu$ l)	$Fl_{SA}$ (20 $\mu$ l)
Total calcium (mmol/l).....	$2.40 \pm 0.13$	$1.95 \pm 0.10$
Supernatant calcium (mmol/l).....	$1.41 \pm 0.05$	$1.26 \pm 0.05$
Estimate percent of nonprotein-bound calcium.....	$59 \pm 3.0$	$65 \pm 4.5$
Protein (g/100 ml).....	$7.0 \pm 0.3$	$3.1 \pm 0.2$
Final pH.....	$7.5 \pm 0.1$	$7.7 \pm 0.3$

<sup>a</sup> Total values are for 12 animals, and supernatant values for 9. The pH figures after ultracentrifugation bear no certain relationship to fresh fluid values.

fluid decreased from 3.1 to 0.1–0.3 g/100 ml following ultracentrifugation. No detectable phosphate was lost from the serum or fluid on ultracentrifugation. The initial level in serum was too low to register the predicted differences due to protein binding.

These data provide two important points of information. First, the high phosphate concentration in  $Fl_{SA}$  cannot be attributed to a mineral phase. Second, it appears that calcium was bound to macromolecular components of the cartilage fluid, probably to the protein, to a greater extent per unit weight than to plasma proteins.

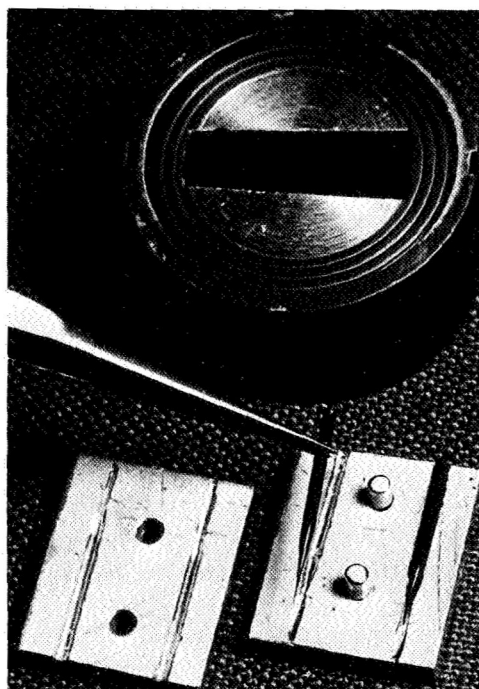


FIGURE 137. Holder for Model L ultracentrifuge and micropipet in position.

TABLE XXVII  
ANALYSES OF SUPERNATANT FRACTIONS OF RACHITIC RAT SERUM AND HYPERTROPHIC  
CELL CARTILAGE FLUID ( $Fl_{SA}$ )<sup>a</sup>

	Serum	$Fl_{SA}$
Number of animals.....	10	10
$Pi_{TCA}$ (mmoles/l).....	$0.65 \pm 0.1$	$1.7 \pm 0.1$
$Pi_{TCA}$ in supernatant (mmoles/l).....	$0.60 \pm 0.1$	$1.7 \pm 0.1$
Nonprotein-bound calcium $\times$ nonprotein-bound $Pi_{TCA}$ ( $M \times 10^{-7}$ ).....	0.91	2.1

<sup>a</sup> Acid-soluble phosphate,  $Pi_{TCA}$ ; supernatant fractions after ultracentrifugation.

URIST: That is very interesting. Dr. McLean, can you interpret these findings for us?

McLEAN: I am afraid not.

URIST: Dr. Howell, how do you interpret the results of these analyses? The chemical composition of the fluid obtained by micropuncture,

which we will call "extracellular fluid," is quite different from an ultrafiltrate of plasma, in chemical composition.

HOWELL: Yes.

PRITCHARD: Is it extracellular fluid?

URIST: It is "extracellular fluid" of cartilage.

PRITCHARD: If you are getting some of the fluid from the cell cytoplasm, would that not have a lot of phosphate in it?

HOWELL: That problem has been a major concern and was partly the reason for measuring chloride, nucleotide, and protein.

FREMONT-SMITH: The high water content of the fluid as compared with plasma would argue very strongly against any significant amount being intracellular fluid, because there you would expect a higher protein content and less water.

HOWELL: Yes.

FREMONT-SMITH: You did not use the freezing point or osmotic pressure?

HOWELL: No.

PECK: Another possibility might be to measure the potassium concentration.

HOWELL: Potassium measurements would be ideal but difficult for us at the present time. Electrometric chloride titration has been a rather easy ancillary technique to have in operation, concurrent with the other methods described.

PRITCHARD: Did you get a lot of contaminated specimens that you did not use?

HOWELL: No; we discard perhaps 5 percent of data based on clear-cut errors of technique, particularly accidental blood aspiration. The hypertrophic cell cartilage is quite transparent and the bony borders readily visualized. Fortuitous puncture of a few cells with a micropipet could hardly account for our findings.

BAUER: Have you made any tracer studies by injecting  $^{32}\text{P}$  intravenously?

HOWELL: Again, the volume is too small. You cannot compute the amount of radioactivity available. You could use that if you had an *in vitro* incubation, but you could never label it heavily enough *in vivo*.

BÉLANGER: So the conclusion is that this particular region of the cartilage is capable of concentrating soluble phosphate, is that it?

HOWELL: With regard to the high  $\text{Fl}_{\text{SA}}/\text{serum}$  ratio of  $\text{Pi}_{\text{TCA}}$ , it remains to be demonstrated whether this represents a physiologic mechanism to elevate inorganic phosphate for calcification or whether it merely reflects slow catabolism of the increased mass of senescent hypertrophic cartilage cells characteristic of rickets. As for the former possibility, the local action of a phosphate transferase (ref. 200) on nucleotides such as ATP might have regulatory effects on calcification,



and the elevated inorganic phosphate may be a reflection of these processes.

In any event, we hope to distinguish between hypothetical mechanisms for regulation of mineral ion transfers in the endochondral plate by further characterization of these microscopic fluid phases, one ( $Fl_{SA}$ ) drawn from a site which potentially or actually calcifies, and the other ( $Fl_{SB}$ ) drawn from an adjacent noncalcifying site.

BÉLANGER: Also, we know that in this particular area, this chondroitin sulfate outside the cell does concentrate calcium which is in chelated form. This we demonstrated histochemically a few years ago by putting demineralized, fixed sections in radioactive calcium and doing autoradiographs (ref. 201). Dr. Migicovsky, in his laboratory, has extracted the chondroitin sulfate from this mass of cartilage and has shown that it could chelate calcium.

MCLEAN: What is the ion product in this?

HOWELL: One may compute an approximate nonprotein-bound calcium  $\times$  phosphate product. For these cartilage fluid samples from untreated severely rachitic animals, the average product was about  $2.1 \times 10^{-7} M$ . (See table XXVII.)

FREMONT-SMITH: Are there any data on pH?

HOWELL: I would rather not be quoted on figures yet, but we have made many measurements and it appears to range from 6.9 to 7.1 in the untreated rachitic cartilage on these samples that can be isolated under oil.

HOLTZER: Is there any comparison with vitreous humor in this kind of stuff?

FREMONT-SMITH: The protein is a good deal higher. In the vitreous humor, the protein is very unlike aqueous humor. Aqueous humor is very low, down to a level of about 40 or 50 milligrams, I think, and this is up to 2 grams; there is a major difference in that respect.

URIST: Aqueous humor is also low in total phosphorus—only about 1 to 2 mg/100 ml.

RAISZ: Do you think it is inorganic phosphate or not? I ask this because there could be a difference in nucleotide content. Was it possible to analyze for any soluble organic phosphates?

HOWELL: We have run ATP and guanosine triphosphate through our analytic system and it does not register as a trichloroacetic acid-soluble phosphate. However, histidine-nitrogen-linked phosphate would be liberated under pH 7. We are unable to distinguish acid-labile from inorganic phosphate in cartilage fluid.

URIST: Let us return to the subject of cellular differentiation and interrogate the experts on induction systems. Perhaps we can find areas of some general agreement. Induction takes place with inter-

action of cells. There are two groups of cells in every induction system: inducing cells and responding cells. In both groups, the cells are proliferating and not resting. We are not able to tell these cells apart or classify them or give them a specific name. Dr. Saxén, can you challenge these simple statements about induction systems?

SAXÉN: I may just add one thing. When you said that there is an inducing cell and a responding cell—we know some model systems where this interaction seems to be mutual. That is, the responding cells are somehow inducing or interacting with the so-called inducing cells. This is seen in the development of the central nervous system, at least.

FREMONT-SMITH: A sort of mutual feedback?

SAXÉN: Yes. They can do both.

URIST: They can function either as responding or inducing cells, is that the idea?

SAXÉN: I would not put it quite that way. Let us say with regard to the inducing cells, their development is somehow dependent on the presence of the responding cells.

HOLTZER: I think "reciprocal" would be a good word, and I do not want to get into semantics, but I must quickly point out that there is vitamin A. Does one need a cell? Vitamin A is the nicest induction system I know. The other point is the homotypic interaction mentioned yesterday, where there is crosstalk between like cells. So, with these provisos, I am sure you can go ahead because it will not reduce the impact, but, I think finally, if we are ever going to get to a model of induction, which I assume we are leading to, we are going to have to contend with a passing remark by Dr. Arnaud yesterday, and that is, "What is a hormone?" I just anticipate this right from the start.

URIST: Yes; but let us first consider the development of the cells that the hormones are going to act upon.

YOUNG: Dr. Urist, some people, such as myself, have taken a general view that all changes in cell specialization probably involve the same intracellular mechanism, and consequently the shift of specialization within normal growing bone would not be different in mechanism from the shift that you might observe in your experimental situation. In that regard, do you agree or disagree that any change in cell specialization represents an induction system?

URIST: I think I would agree that induction systems bring about differentiation of specialized cells.

YOUNG: So that if one of the precursor cells in bone becomes an osteoblast, that is also an induction?

YOUNG: I think it is important to emphasize that the complexity of this reaction is probably within the cell and involves the selective activation and repression of integrated groups of genes, and that there is no

reason to think that this mechanism differs either prenatally or postnatally or even in unicellular or multicellular organisms or *in vitro* or *in vivo*.

URIST: We will try to discuss the question of genetic mechanisms later. The question is, assuming that there is an inducing cell or tissue, does an inducer exist? The literature defines inducers as many different kinds of things, such as small protein molecules, changes in oxygen saturation, CO<sub>2</sub> tension, mechanical factors, pressure or stress and strain. Thus, an inducer may be something from the outside which is a chemical entity, possibly a lipoprotein, a mucoprotein, or a protein-protein complex; it may be a change in intercellular or intracellular oxygen saturation and CO<sub>2</sub> tension.

If we define an inducer as something which may have any one of these forms, then it is reasonable to assume that inducers do exist.

HOLTZER: You must mention inorganic ions to make the list reasonably complete.

URIST: Let us add inorganic ions, endocrine factors, metabolites, antimetabolites, and activators. In the broadest sense a hormone is an activator.

PRITCHARD: I thought it was a chemical messenger.

FREMONT-SMITH: "I arouse to action: *hormao*." So an activator I think is all right. It is a messenger which activates.

URIST: The word "activator" is used by molecular biologists who are changing the language of cell physiology to make it broad in perspective and applicable to systems *in vitro* (ref. 202).

PRITCHARD: If you now change from discussing what goes from one cell to another to what happens when it gets there, which is a different concept—

URIST: If we define an inducer as something as broad in its nature as the factors in all of these categories, could everyone agree that an inducer exists? Do you have any objection to that?

PRITCHARD: I do not think we need to be too specific. Some vehicle for transmission of information from one cell to another is all that is necessary.

URIST: What would the vehicle convey?

PRITCHARD: Some vehicle for transmitting information from one cell to another.

URIST: How would you define the word "vehicle"?

PRITCHARD: Some means of getting a message across. It might be electrical, physical, chemical.

URIST: Let us add the effects of electrical stimuli. Is there anything else you can add?

PRITCHARD: Any other channel of communication anybody can think of.

URIST: Let us add "unknown factors."

YOUNG: I would like to suggest that most inductive reactions—that is, changes in cell specialization—may be traced back to a change in the microenvironment of the reacting cell (refs. 111, 203, and 204). Where we have some information on what seem to be the critical aspects within this admittedly complex microenvironment, it appears that they fall in this sort of category, where we see that the critical agent has very little inherent specificity or complexity in itself, again emphasizing that the complexity is in the reacting system.

URIST: I have drawn a diagram of a cell. The cell membrane appears as a broken line to show that there is communication with the outside world; the same applies to the nuclear membrane.

FREMONT-SMITH: I think nature broke it for you, but I am glad that you copied nature.

URIST: Diagrammatically, we are dealing with an open system.

FREMONT-SMITH: You mean a partly closed system.

URIST: Yes; assuming that inducers exist, defining the inducer as broadly as possible, where is it located? Is the inducer intracellular or is it extracellular? Since induction systems represent systems in which there is interaction of cells, can we assume that the inducer is cellbound and transported by cells?

PRITCHARD: What is the inducing cell and what is the responding cell?

URIST: The progeny of a young chondrocyte in the germinal layer of the epiphyseal cartilage are inducing cells. The evidence is that if you transplant a piece of cartilage that has germinal layer cells, these cells do not go on and make more cartilage but they produce chondrolysis, interact with the ingrowing cells of the host bed (the mesenchymal or responding cells), and where this interaction occurs you see an induction system for bone formation.

HOLTZER: But is it not fair to point out that in fact you do not know the source of that bone cell? There is no rigorous demonstration that this particular osteocyte can trace its lineage back to either what you could call the inducing system or the responding system. It is there.

URIST: You are correct. We cannot say whether or not a cell came from the host bed or whether it came from the young modulated chondrocyte. What I observed was that there was: (1) cell proliferation, (2) mixing of cells from two sources, and (3) differentiation of bone in excavation chambers in which the cells are swarming.

OWEN: Marijke Holtrop (ref. 205) has shown that at least some of them come from the cartilage cells.

URIST: The modulated cartilage cells can modulate and become osteoblasts.

RAISZ: And also host cells became osteoblasts in the same preparation.

OWEN: The cartilage cells were labeled before transplanting the piece of cartilage and after transplantation labeled osteoblasts and osteocytes were found.

URIST: We also labeled the cartilage cells and found a few labeled osteocytes. Our observations of callus and articular cartilage suggest that a very small number of osteocytes may be linear descendants of modulated labeled chondrocytes and that modulated chondrocytes may act under special circumstances as responding as well as inducing cells. The source of the induced cell is immaterial. The important thing is whether the pathway of the cell has been altered by interaction with other cells in an excavation chamber. The following five tables present data on transplants of articular cartilage labeled with  $^3\text{H}$ -thymidine for nuclei and  $^3\text{H}$ -glycine for matrix. The matrix label enables one to distinguish transplanted cartilage from new cartilage formed after transplantation.

TABLE XXVIII  
FATE OF  $^3\text{H}$ -THYMIDINE LABELED CHONDROCYTES AFTER TRANSPLANTATION OF  
ARTICULAR CARTILAGE INTO ANTERIOR CHAMBER

Number of implants	Days in anterior chamber	Cells counted	Cells labeled		Grain count > 50 or < 50	Percent of labeled cells in specific cell type		
			Total	Percent		Round	Hypertrophic	Squamous
1.....	1	5107	142	2.78	>	100	0	0
					<	98	2	0
1.....	3	1639	22	1.34	>	86	0	14
					<	100	0	0
1.....	4	2043	3	.15	>	100	0	0
					<	100	0	0
1.....	5	3159	10	.32	>			
					<	90	0	10
1.....	6	1596	14	.88	>	0	100	0
					<	87.5	0	12.5
1.....	8	1190	6	.50	>	66.6	0	33.3
					<	100	0	0
4.....	18	6241	32	.43	>			
					<	100	0	0
5.....	28	2912	6	.21	>	100	0	0
					<	100	0	0

Table XXVIII illustrates the percentage of labeled cells in transplants of articular cartilage at various intervals of from 1 to 28 days. The labeled cells were chiefly round chondrocytes of the germinal layer at all intervals after transplantation. This suggests that, following transplantation, the cells reduce the rate of further development of

cartilage and assume the pathway of development of cells for bone induction.

Table XXIX shows the relative constant proportion of round (zone B) to hypertrophic to squamous (zone A) cells in double-labeled nuclei and matrix transplants before and after bone induction. This observation affirms the fact that transplantation suppresses further proliferation of articular cartilage.

TABLE XXIX  
FATE OF CHONDROCYTES WITH  $^3\text{H}$ -THYMIDINE LABELED NUCLEI AND  $^3\text{H}$ -GLYCINE LABELED MATRIX IN ARTICULAR CARTILAGE TRANSPLANTS

Number of implants	Days in anterior chamber	Cells counted	Cells labeled		Percent of labeled cells in specific cell type		
			Total	Percent	Round	Hypertrophic	Squamous
7.....	14	26 267	331	1.26	93	4.8	2.2
10.....	28	20 177	428	2.12	89.7	9.3	1
14.....	35	25 518	336	1.32	91.4	3.8	4.8

Table XXX lists the progressive reduction in the percentage of labeled cells, presumably modulated chondrocytes, in excavation chambers produced by the host in transplants of articular cartilage. The decrease in the number of cells with high grain counts and the increase in the number of cells with low grain counts indicate that chondrocytes modulate and undergo mitotic division inside the excavation chambers.

TABLE XXX  
FATE OF EXCAVATION CHAMBER CELLS OF ARTICULAR CARTILAGE LABELED WITH  $^3\text{H}$ -THYMIDINE AND  $^3\text{H}$ -GLYCINE BEFORE AND AFTER BONE INDUCTION

Number of implants	Days in anterior chamber	Cells counted		Grains counted		
		Total	Percent	> 50	≤ 50	Average
7.....	14	1 130	7.7	1	87	10.6
10.....	28	16 684	2.4	42	351	14.3
14.....	35	24 770	1.1	35	240	10.7

Table XXXI summarizes counts of labeled bone cells produced from transplants of double-labeled articular cartilage cells. The percentage

of labeled osteoblasts and osteocytes was relatively low, but the average grain counts were at least 10 percent of maximum. These data suggest that a small, indeterminate number of the bone cells are linear descendants of modulated chondrocytes.

TABLE XXXI

FATE OF CELLS OF ARTICULAR CARTILAGE TRANSPLANTS LABELED WITH  $^3\text{H}$ -THYMIDINE AND  $^3\text{H}$ -GLYCINE, OUTSIDE OF EXCAVATION CHAMBERS OR AREAS OF BONE INDUCTION

Number of implants	Days in anterior chamber	Osteoblasts			Osteocytes			
		Total counted	Percent labeled	Average grain count	'Total counted	Number labeled <sup>a</sup>		
						Total	Percent	Average grain count
7.....	14	0	0	0	0	0	0	0
10.....	28	6 788	.13	7.3	5 239	34	.6	10.5
14.....	35	13 050	.05	15.2	10 866	44	.4	9.9

<sup>a</sup> All labeled cells had 50 grains.

Table XXXII summarizes grain counts of chondrocytes in articular cartilage transplants. These cells always had high grain counts, gave the appearance of cartilage cells transforming directly into bone cells, and consisted mainly of inclusions of unabsorbed cartilage in the middle of an island of bone tissue.

TABLE XXXII

FATE OF CELLS OF ARTICULAR CARTILAGE TRANSPLANTS LABELED WITH  $^3\text{H}$ -THYMIDINE AND  $^3\text{H}$ -GLYCINE

Number of implants	Days in anterior chamber	Chondroid		
		Total counted	Number labeled <sup>a</sup>	Percent
7.....	14	11 631	43	0.37
10.....	28	3 990	37	.93
14.....	35	1 588	13	.82

<sup>a</sup> All labeled cells had > 50 grains.

YOUNG: If we agree that the mechanism is the same, it does not matter for the purposes of this discussion which cells were induced.

URIST: But the point is that the progeny of these cells are not inducing the old adults. They are inducing the young, new generations

TABLE XXXIII  
INDUCTION SYSTEMS FOR OSTEOGENESIS, CHONDROGENESIS, AND MYELOGENESIS IN ARTICULAR CARTILAGE TRANSPLANTS

Time, weeks	Inducing cell type	Responding cell type	Hypothetical changes locally in microenvironment	Responding cell	Induced cell
0.....	Germinal chondrocyte zone B ( )				
2.....	↓ Resorption of cartilage matrix 1st mitotic division	↓ Perivascular connective tissue cell of host, several mitotic divisions			
3.....	↓ Rapidly dividing young connective tissue cells in excavation chambers	↓ Perivascular connective tissue cell of host, several mitotic divisions	Low CO <sub>2</sub> tension, high O <sub>2</sub> saturation, compaction	Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	
4.....	↓ Rapidly dividing young connective tissue cells in excavation chambers	↓ Perivascular connective tissue cell of host, several mitotic divisions		↓ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	Osteoprogenitor cell, osteoblasts, osteocytes
4.....	↓ Rapidly dividing young connective tissue cells in excavation chambers	↓ Perivascular connective tissue cell of host, several mitotic divisions	High CO <sub>2</sub> tension, low O <sub>2</sub> saturation, compaction	↓ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	New chondroprogenitor cell, chondrocytes, chondroblasts
4.....	↓ Rapidly dividing young connective tissue cells in excavation chambers	↓ Perivascular connective tissue cell of host, several mitotic divisions	Normal O <sub>2</sub> tension	↓ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	Fibroprogenitor cells, fibroblasts, fibrous tissue
6.....	↓ Rapidly dividing young connective tissue cells in excavation chambers	↓ Perivascular connective tissue cell of host, several mitotic divisions	Low CO <sub>2</sub> saturation, low pressure		
6.....	↓ Rapidly dividing young connective tissue cells in excavation chambers	↓ Perivascular connective tissue cell of host, several mitotic divisions	High O <sub>2</sub> saturation, low CO <sub>2</sub> saturation, low pressure	Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	Hemopoietic stem cell, hematoblasts, bone marrow



of cells, and the process of bone induction may take place during a period of one or more mitotic divisions. Table XXXIII is a schematic representation of the time sequence and the hypothetical changes in the microenvironment of a transplant of articular cartilage, associated with the appearance and development of new fibrous tissue, bone, cartilage, and bone marrow. Similar to embryonic induction systems, for all kinds of organs, bone-induction systems produce many different kinds of cells concurrently or in a sequence in addition to osteoblasts and osteocytes.

PRITCHARD: It might be worthwhile to drop the word "induction" altogether.

URIST: It might be worth considering all the evidence in its favor first.

PRITCHARD: You have two populations merging.

URIST: Yes.

PRITCHARD: As a result, certain things happen which lead to bone formation. Some of the cells of that mixed population apparently become osteoblasts. That is all you have really demonstrated. The rest is hypothesis.

URIST: You cannot mean all we have demonstrated. You may mean what we have demonstrated.

PRITCHARD: All—in this system.

URIST: Before we discard the word "induction," let us see if we can use it; if not, we will be glad to dispose of it.

PRITCHARD: It could be a handicap—the language.

FREMONT-SMITH: If the word "induction" does give you a theory that provides experiments to show you whether or not the theory is correct. There is a great advantage in theory, because it induces experiments, and we are looking for the induction of certain experiments as a result of theoretic development.

URIST: We are perfectly willing to throw out that word and the whole concept of induction, but before we do that we must have a reason.

PRITCHARD: Well, the fact that there is so much confusion and so little evidence as to what these transmitting agents are——

URIST: Some of the confusion is in the mind of the possessor, and some of it is not. Let us go on, and perhaps we can see more evidence.

PRITCHARD: All right.

SAXÉN: I have the very same impression, that people usually do not like the words, "induction," "inducer." It somehow seems to irritate people.

FREMONT-SMITH: That means it makes them uncomfortable.

SAXÉN: That may be. Some years ago, in a meeting such as this, somebody suggested that instead of speaking of induction we might

say that something provides conditions necessary for a certain type of differentiation situation. It is easier to use the word "induction," but we really do not always refer to such specific approaches, and all this that has been listed might just be a kind of proper environment, proper condition for a subsequent development.

URIST: Yes; the inductor may be a highly complex list of chemical, physical, electrical, and mechanical influences. As a matter of fact, it could be an entity as complex as life itself.

NICHOLS: How rigorous is the evidence that no cells can transmute from one type to another without cell division?

URIST: In bone-induction systems, cell division precedes differentiation of cells into osteoblasts.

NICHOLS: I asked you, How rigorous is the evidence? In a sense, Marijke Holtrop's experiments (ref. 206) suggest that cell division is not a prerequisite for the process.

URIST: I am uncertain about whether mitosis is always a prerequisite.

NICHOLS: The question is, Must there be cell division in order to get specialization?

URIST: When you say "must," you confront us with the problem of interpreting exceptions to the rule that mitosis precedes differentiation of osteoprogenitor cells.

NICHOLS: That is what I meant.

URIST: When you say "must," you raise a different kind of question; the plain fact, however, is that preparation for mitosis is the basis of uptake of tritiated thymidine, and the labeling of osteoprogenitor cells.

HOLTZER: I would like to hear a little more about these experiments. I do not know they work at all. Has somebody labeled a cartilage cell and observed it transforming into an osteoblast without undergoing a cell division?

OWEN: I feel that the work is not conclusive on this question because, as far as I know, grain-count studies have not been done, although they may have been done recently. In this case, therefore, it is possible for a labeled cartilage cell to have gone through one or more divisions before it is seen as a labeled osteoblast, and so forth.

HOLTZER: This is an important point.

NICHOLS: I think some of this depends on how we are going to define "induction."

URIST: We are going to try to define "induction" on the basis of experiments upon material in which there is always clear-cut evidence of cell division before there is cellular differentiation. The occurrence of bone formation in small amounts by an ancillary process is an exceptional situation and does not permit us to dispose of the occurrence

of bone formation by the process of induction of rapidly dividing cells in large numbers in a typical situation.

BÉLANGER: What else do you see?

HOLTZER: Are you going to accept "osteoblast" and "osteoclast" as "induction"?

YOUNG: My opinion is that basically the intracellular rearrangements must be the same. The cell is not set up to have six or seven different ways of changing its specialization depending on the experimental situation.

NICHOLS: Some of us do not know that that happens.

HOLTZER: Actually, I agree with you and I hope you are right, but in point of fact, nobody has really rigorously eliminated the possibility of some kind of other intracellular rearrangement.

URIST: Are you raising the question of whether or not one specialized cell form can turn into another specialized cell form without either mitotic division or modulation? In the older literature, this was termed "metaplasia." We can discuss that subject after we examine the role of mitotic division in a typical bone-induction system.

BÉLANGER: May I say just one thing? This is with reference to the observations of Dr. Holtrop (ref. 205). I have seen her material and I have read her thesis. The only evidence, to my mind, is that in some of these empty lacunae of the hypertrophic cartilage, there are cells which look like osteoblasts and which are labeled. Now, we know that in this particular area the cartilage cells die. Is it not possible that as they die in large numbers and they have labeled nuclei, the precursors of the osteoblasts that are just coming in might utilize this material to make their own DNA? Is this not possible?

OWEN: I think it is very unlikely, although possible.

BÉLANGER: Even in a system such as this, where there is a concentration locally? This is in culture, mind you; this is not *in vivo*. I just want to make the point that just interpreting such important phenomena on purely morphologic data is dangerous.

URIST: Here we bring into evidence the results of an experiment that we know about only partially.<sup>1</sup>

PRITCHARD: Dr. Urist, you are making a great point about mitosis, and I am wondering why you are stressing mitosis as being such an important aspect of your theory. Is it essential?

URIST: I have not given my theory yet.

PRITCHARD: You are leading up to it.

URIST: A definition of the term "induction" is a mechanism of

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<sup>1</sup> Holtrop (ref. 206) concluded: "Cartilage cells probably first differentiate into connective tissue cells, periosteum or perichondrium, and then bone afterwards."

cellular differentiation that occurs from the interaction of two cells, an inducing and a responding cell, as a result of which the latter follows a pathway of development it otherwise would not have exhibited. I have not proposed a theory, but I assume that mitosis occurs before, during, or after induction. In every bone-induction system I have seen there is a preliminary stage of cellular proliferation. The inducing and responding cells are the progeny of two different preexisting groups of cells.

HOLTZER: I do not think there is any question about this. If one goes through the literature on induction, mitosis is certainly there. In the sense of a purist, I was a little shocked at the unequivocal usage of this particular experiment as evidence against mitosis.

OWEN: No; I think you have misunderstood what has been said.

HOLTZER: I would say there is not a single induction experiment that, to my knowledge at least, does not involve mitotic activity.

PRITCHARD: Do you mean mitotic activity immediately preceding induction? I have observed bone and cartilage formation in a repaired tendon several weeks after the burst of mitotic activity which follows tenotomy. I had the impression that the tendon cells became transformed into bone and cartilage cells without any mitosis in between.

URIST: Have you described tendon cells turning directly into bone cells?

PRITCHARD: It was well described by Buck (ref. 207) in 1953. Turkey tendons ossify, I believe, without mitosis. The formation of bone does not necessarily imply the intervention of some mysterious induction system, nor is it necessarily preceded by mitotic activity. In the normal development of the skeleton, a lot of tendon is converted into bone without the cells undergoing mitotic division. Presumably, something induces the change, but whatever it is it does not have to cause the cells to divide first.

URIST: You have cartilage cells in tendon insertions that may be inducing cells that are partly induced in the direction of osteogenesis.<sup>2</sup> Ossification of the tendon is associated with an interaction of cells, and it is a process that takes place like an infectious wave that moves through the tendon. There are systems in which it would appear as though one specialized cell form transforms directly into a bone cell

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<sup>2</sup> Buck (ref. 207) described multiplication of new cells of the *peritenonium internum* inside the cut ends of the tendon, and noted that it was unlikely that mature tendon cells undergo mitosis. He found bone at the junction of the new cells with the old stumps of tendon, but not until about the third month after the tenotomy. Buck thought that the new bone was closely associated with cartilage-like cells in the area of repair, and wrote that cartilage and bone may represent metaplasia of fibroblasts in the regenerating parts (not the previously existing parts) of the stumps of the tendon.

without modulation. This occurs in the deer's antler, but the tissue is referred to as "chondroid," because it looks like cartilage but is not true cartilage. The way it happens is something like in birds in which the cartilage cells appear to transform into bone cells. Nevertheless, in the process, cells proliferate along an edge, blood vessels sprout, periosteum-like and endosteum-like tissues are involved, and many undifferentiated cells are recruited all along the way to differentiate first into osteoblasts and then into osteocytes. If a specialized cell appears subjectively to transform directly into an osteocyte, it is necessary to consider its past history and possible effects as an inducer as well as a transformer.

PRITCHARD: Well, we have been studying tendon repair for the past 2 years, and we have been looking at tendon and ligament attachments to bone. Transitions from tendon cells to bone cells are perfectly clear.

URIST: Please present the evidence for this view.

PRITCHARD: I thought it was very well known.

URIST: The process of ossification of tendon may give one the impression that tendon cells transform into bone cells, and this could be a side issue. The mainstream of cell differentiation comes from responding cells around ingrowing capillary sprouts. Transforming or modulating tendon cells may be inducers.

SAXÉN: I agree completely with what Dr. Holtzer said. It is very easy to design an experiment to study mitosis. You combine the responding tissue with an inducer, and the control with another tissue which does not lead to morphogenesis in the responding cell. One of the very first changes in the induced cells is an increased uptake of thymidine.

URIST: Yes.

SAXÉN: Whereas in the other case, where the cells survive, you do not see this.

HOLTZER: Just for the record, for the past 2 years we have been studying the necessity for mitosis in the two induction systems that we have been working with for many years.

FREMONT-SMITH: You found it necessary?

HOLTZER: Yes.

URIST: To observe the phase of mitosis in an induction system, a cell-labeling technique is often helpful.

PRITCHARD: How do you know I have not done this?

URIST: I have read the articles by Pritchard and associates on the cells of the blastema in healing fractures in vertebrates in unlabeled histologic section.

PRITCHARD: That is not all I have written.

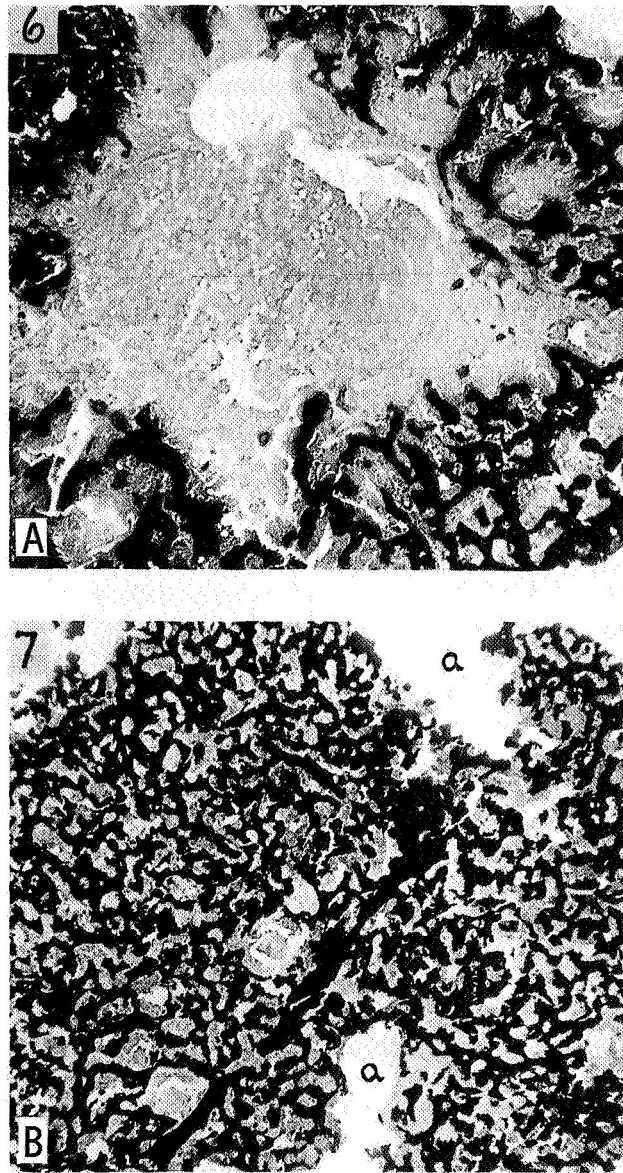


FIGURE 138. Photomicrographs of undecalcified femoral marrow of pigeons. (a) Section from a pigeon with a 6-millimeter follicle in ovary; the pale-staining central core of hemopoiesis marrow is in sharp contrast to the black trabeculae of medullary bone. (b) Section from a pigeon with a 20-millimeter follicle in ovary; the bone has permeated the entire marrow. The gaps marked *a* are the result of penetration of the marrow by the dental drill used to remove the dense cortical bone. Fixation: 10 percent neutral formalin; von Kossa stain. 35 $\times$ . [From Bloom et al., ref. 175; reprinted with permission of the publisher.]

MCLEAN: Would you like a clear-cut demonstration of this induction process?

URIST: Yes; we need some tangible material to discuss the subject of mitosis.

MCLEAN: This is the system of endosteal bone formation, medullary bone formation, in the pigeon, without any external influence. This is what the pigeon does.

In figure 138(a), bone is growing out into the marrow cavity. This reaction is initiated in the endosteum, and it proceeds in a wave out into the marrow cavity until, as you see in figure 138(b), it has filled the marrow cavity with new bone. This is the gross phenomenon.

Figure 139 shows what is going on in this process. At the bottom there is a small spicule of bone which has formed at the endosteum, and the wave proceeding up into the marrow with abundant mitoses can be seen. The cells, the reticular cells, are in the process of being transformed into bone cells. This work was done by Bloom et al. (ref. 175), and it is as clear a demonstration of what we are talking about as I know of, because we can see the wave as it progresses into the marrow, and the marrow reticular cells are being drawn into the wave by what we are calling induction. Do you agree with that?

URIST: Yes. And there is abundant cell division and proliferation as this process goes on.

MCLEAN: It is full of it.

PRITCHARD: Can I describe my induction system? I think it is fundamental and should not be discussed as being wrong because it was done by a different histologic method.

URIST: If you have evidence, it will not be dismissed or undervalued; please present it.

PRITCHARD: This is the induction system that we have been working on. If you take the tendo Achilles of a rat, young or old, cut it cleanly and let it repair by itself, in 40 to 70 days you will invariably find that bone has developed in the tendon, not only in the new tendon, but also in the old tendon ends. There is a long lag period.

URIST: How long?

PRITCHARD: Up to 40 days after operation. In the first few days, there is a lot of mitotic activity. After that, the cells are engaged in collagen production, and during the maturation of the new tendon you do not find mitotic figures.

URIST: How do you define the term "maturation of the tendon"?

PRITCHARD: During the maturation process, the collagen fibers become thicker and more densely packed and the cells get flatter—the sort of thing you would expect in any repair.

URIST: What is the origin of the cells?

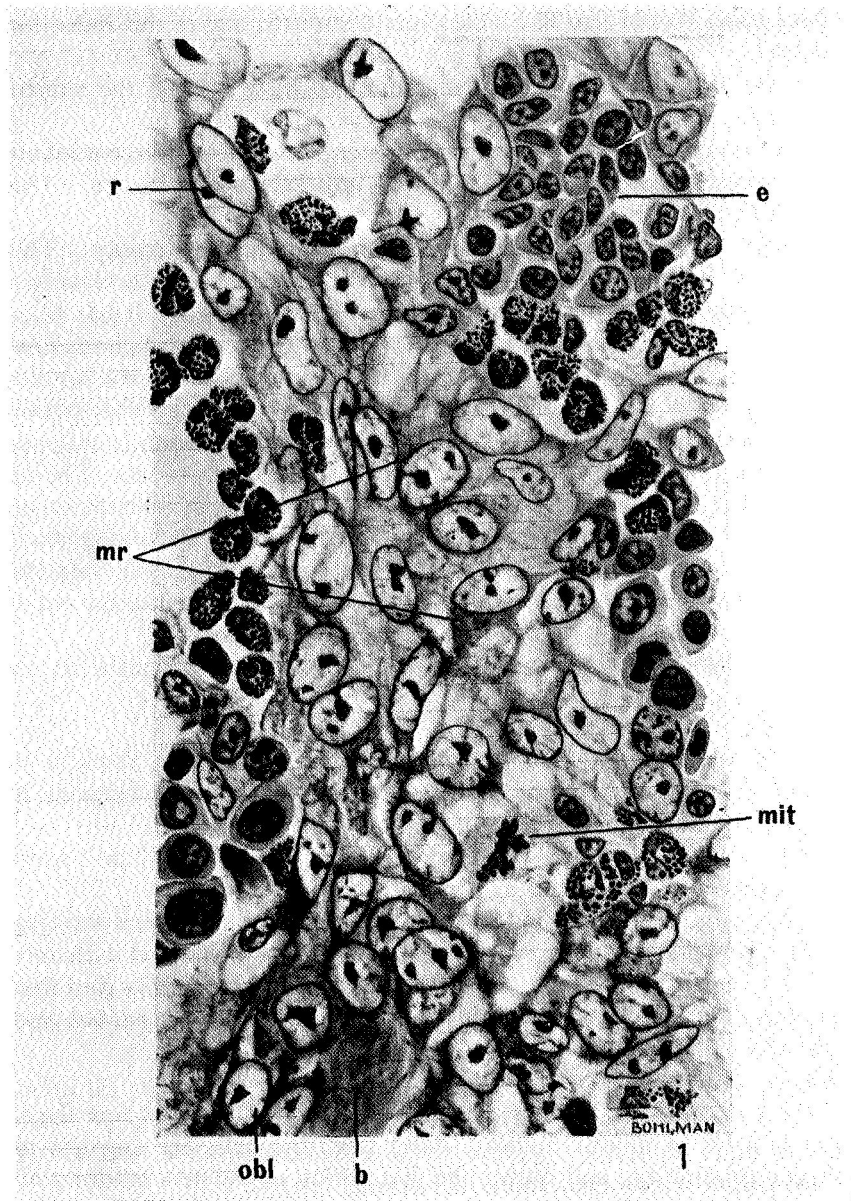


FIGURE 139. Photomicrograph of section of bone marrow from pigeon with 6-millimeter follicle in ovary, showing changes in reticular cells (r) and osteoblasts (obl), ahead of the advancing trabecula of bone (b); mobilizing reticular cells (mr); mitosis of reticular cell (mit); erythropoiesis (e). Zenker-formol fixation; HEA stain.  $\times 1650$ . [From Bloom et al., ref. 175; reprinted by permission of the publisher.]



PRITCHARD: These cells come largely from the epitenon, but some come from the tendon ends, because one sees mitotic figures in the tendon ends. If the operation is done roughly, they also come from the paratenon.

URIST: How many generations of cells or cell divisions were derived from cells of the paratenon?

PRITCHARD: From the early——

URIST: How many cell divisions occurred in the time of your experiment? You cut the tendon?

PRITCHARD: Yes.

URIST: There was an ingrowth of cells from the tendon sheath. How many cell divisions occurred in 40 days?

PRITCHARD: There probably were many to begin with. In any repair process, mitotic activity is concentrated in the first few days; after that, the formation of collagen is the predominant feature. I think this has been displayed so often in repair processes that I do not have to prove it. After this time, when, to all intents and purposes, you have a tendon that is mature, you begin to see these changes in the tendon without any sign of mitosis.

FREMONT-SMITH: Without any further signs of mitosis.

PRITCHARD: Oh, all right, but any tissue in the body has had a long history of mitotic activity.

FREMONT-SMITH: But the mitosis that took place here took place as a result of the trauma, and not without any further mitosis.

PRITCHARD: Without any further mitosis these cells changed their form, and their matrix became calcified.

URIST: Does the new tendon come from new fibrous connective tissue and is this new tissue fibrous tissue or tendon?

PRITCHARD: It is somewhere between fibrous connective tissue and tendon. You know tendon never repairs perfectly.

URIST: The end product of repair of a cut tendon is a scar or irregular meshwork of fibrous tissue; the fibers are not organized in parallel bundles like the original tendon.

PRITCHARD: A repaired tendon with the fibers parallel is different from a scar with irregular fibers all over the place. It is new tissue that has matured and it is getting toward the tendon state, and if it is left for a year it gets even more like normal tendon.

HOLTZER: I wonder if I might try to resolve this. We might want to go on to something a little more interesting. Clearly, cells do divide and they have an evolution. I think this is the point. You take a cartilage cell, and after a while keratosulfate, for example, comes into the system. This is part of the normal repertoire of an aging cartilage cell. This is one of the things about aging. A cell gradually—very often——

PRITCHARD: I think this is well known, too. All I am suggesting is that a cell which has spent part of its life as a tendon cell can become a bone cell without having to go back to square 1 and revert.

Figure 140 shows a repaired tendon and demonstrates the transition zone—the tidemark where tendon stops and bone begins. There are lines and lines of cells going from the tendon into the bone without mitosis.



FIGURE 140. Photomicrograph of patches of ectopic bone in repaired tendo Achilles of the rat 100 days after tenotomy. 220X.

FREMONT-SMITH: At that time.

PRITCHARD: Well, yes; at that time.

FREMONT-SMITH: But they did have new mitoses just a few weeks before. So the issue here, as I see it, and I really think it is quite clear, is: How long after mitosis can a cell produce bone and not be considered related to the previous mitosis?

PRITCHARD: Plus one other point. While it is in this postmitotic state, can it produce collagen before it goes on to finish the job of making bone? I think it can.

URIST: May I attempt to explain the sequence of events that led up to this picture? Please interrupt and correct any erroneous statements. Tendon is covered with tendon sheath. After the tendon is cut, muscle contraction pulls the ends of tendon apart and creates a space.

PRITCHARD: About 1 centimeter.

URIST: That 1-centimeter space becomes filled with fibrinous clot. The tendon sheath has a capillary circulation and is the source of sprouting capillaries.

PRITCHARD: What do you mean by the tendon sheath? You and I may not be referring to the same thing.

URIST: It is either epitenon or paratenon.

PRITCHARD: Let us take the epitenon, not the paratenon.

URIST: It is a source of sprouting capillaries. First inflammation, then sprouting capillaries, and ingrowth of hundreds, of thousands, of new cells, until a bridge of connective tissue forms between the ends of the cut tendon.

PRITCHARD: I do not like this model. It is not like that, really.

URIST: You said the gap was 1 centimeter in length. What occupied the gap after a week?

PRITCHARD: The sides collapsed.

URIST: Does the original tendon sheath fill the space?

PRITCHARD: I will have to draw it, Dr. Urist, to make my point clear (fig. 141).

URIST: If the sheath collapsed, it would soon reexpand from ingrowth of new cells.

PRITCHARD: But you are guessing at something——

URIST: I have seen hundreds of tendons heal, and the ends are always enclosed in a fusiform mass of tumorlike fibrous connective tissue. The end product of repair develops from a mass of new cells.

FREMONT-SMITH: Why not let Dr. Pritchard explain his figure?

URIST: Please consider the possibility that the ingrowing mass of new cells are the responding cells of an induction system. We cannot underestimate the importance of that pathway of repair. Specialized cells such as cartilage and bone develop from the interaction of the progeny of resident and ingrowing cells in the area of the tendon gap.

PRITCHARD: You have seen two of my figures (figs. 140 and 141), and I have a story to explain; I would like to tell what really happened. I do not really want to take a lot of the audience's time, but I have been challenged on this.

After cutting the tendon, the ends with their epitenon retract, leaving a 1-centimeter retraction gap. The paratenon does not retract, but tends to collapse into the retraction gap. There is usually very little bleeding inside the empty segment of paratenon.

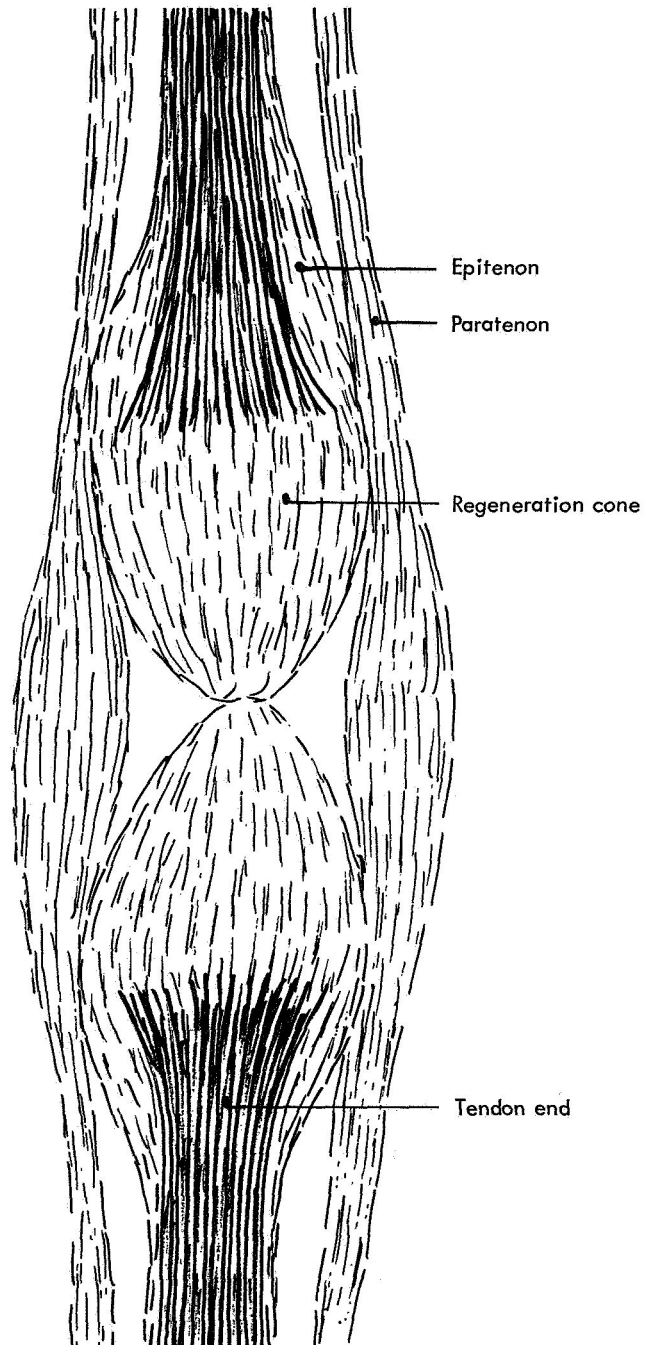


FIGURE 141. Diagram illustrating formation of regeneration cones from tendon ends after tenotomy in the rat.

The epitenon cells, during the next few days, proliferate enormously and form regeneration blastemas which push their way toward each other inside the paratenon tube. They fuse to form the basis of the new tendon which will develop to bridge the gap in the old tendon.

FREMONT-SMITH: Will there be new capillaries in there?

PRITCHARD: Oh, yes; but the capillaries do not come first.

FREMONT-SMITH: They are all together now as it is joining?

PRITCHARD: If the tendon is cut cleanly, the regeneration blastemas come from the epitenon. However, if the operation is less expertly performed, there is proliferation of a paratenon as well, and the new segment of tendon is derived from a complex of paratenon and epitenon cells. After 3 or 4 days, mitosis can be observed in the tendon ends, and cells apparently come out of the end of the tendon and join the blastema.

After 10 days, the tendon ends are grossly swollen and they are enveloped in big cones of new tissue. Now collagen formation, rather than mitosis, is the order of the day. From 30 to 40 days later, there is a functionally useful tendon again, capable of moving up and down within a new sheath. New and old tendon are so perfectly joined that the line of fusion can hardly be made out. Then, in various places, we begin to get bone and cartilage formation. The tendon is transformed into bone and cartilage. This is not confined to the new tendon but occurs in the old tendon as well.

Up to 40 days, you do not find bone or cartilage, but at 70 days you always find one or the other, or both, and along the boundary line between tendon and bone or cartilage you do not find the sort of vascular or mitotic activity you might expect if there were great cellular turnover and change. It is a quiet-looking region where tendon cells grade into bone cells.

URIST: Is it unreasonable to use the term "differentiation"? Is it incorrect to assume that the myriad of new cells in healing tendon, not the small limited number of old tendon cells, would differentiate into bone? Is it not more reasonable to assume that new cells were induced to enter a new pathway of development and specialization?

PRITCHARD: By what?

URIST: By local factors present at an earlier stage and immediately after cell division and proliferation.<sup>3</sup>

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<sup>3</sup> If the process is brought about by alterations in the regulatory genes, the time for bringing these alterations about could be immediately after mitosis during the period of high activity of the nucleolus—the organelle that receives certain species of RNA from chromatin and sends it forth to be clothed in ribosomal protein. The nucleolus is the engine of ribosomal protein synthesis and of ribosomal assemblage, according to Bonner (ref. 208).

PRITCHARD: I do not know how you can know that, especially as this phenomenon is peculiar to the tendo Achilles of the rat.

URIST: The rat is not an exception. It also happens in man.<sup>4</sup>

PRITCHARD: It happened to John Hunter's tendo Achilles. But surely, you are straining the concept of induction a bit far.

URIST: The cells that differentiated into bone could have developed their competence to specialize as osteoblasts at an earlier state of the process of repair.

FREMONT-SMITH: The whole question is one of timing. You agree that mitosis did take place earlier. Dr. Urist says mitosis is important for induction, and then you got bone, and all Dr. Pritchard is insisting on, quite correctly, I think, is that there was not any very active mitosis just before the bone was formed, and Dr. Urist says there was a lot of active mitosis earlier.

CURREY: Which resulted in tendon cells.

PRITCHARD: Which later resulted in bone cells. That is the point. There is an intermediary stage of maturity between the mitotic state and the bone-cell state. If the cells remained immature for several weeks, it would be different.

FREMONT-SMITH: The question is: How long can you have a delay and still call it induction? This is really what the argument is about.

URIST: Osteoblasts do not undergo mitosis. At the stage of development of osteoblasts, the induction system has reached the stage in which mitosis is not an essential feature. Before osteoblasts appear and bone induction can occur, cellular proliferation is a requirement. A supply of new cells, both from inside and outside the tendon, seems essential to account for the large number of new cells—osteoblasts, hemocytoblasts, fibroblasts, and fat cells in a deposit of bone in tendon.

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<sup>4</sup> It can be demonstrated in a specimen from a patient I treated for ossification of the tendo Achilles, as shown in figures 142 and 143. When bone formation occurs, many of the new trabeculae are aligned parallel to the long axis of the fibers of the original tendon, but some are formed also at right angles (fig. 142). In figure 143, new cartilage and fibrocartilage at the top of the figure, new bone at the bottom of the figure around an excavation chamber filled with osteoblasts, osteoprogenitor cells, and sprouting blood vessels at the center suggest that the ossification of the tendon occurs by replacement of new tissue and new cells, not transformation of old cells of the original tendon. If an induction system is set up, the responding or induced cells, not old tendon cells, are the chief source of the new bone. If it is argued that the fibrocartilage came from old tendon cells, there are numerous paired cells to suggest that mitotic division also occurred and continues to occur for some time before and during the osteogenic reaction. Therefore, the evidence is that mitosis must be an integral part of the process of bone induction by tendon.

Hirsch and Morgan (ref. 209) reviewed the literature on the subject of ossification of tendon and pointed out that the theory of metaplasia is unnecessary because the new bone forms in the presence of proliferating fibroblasts, fibrocartilage and cartilage, the same as in endochondral ossification.

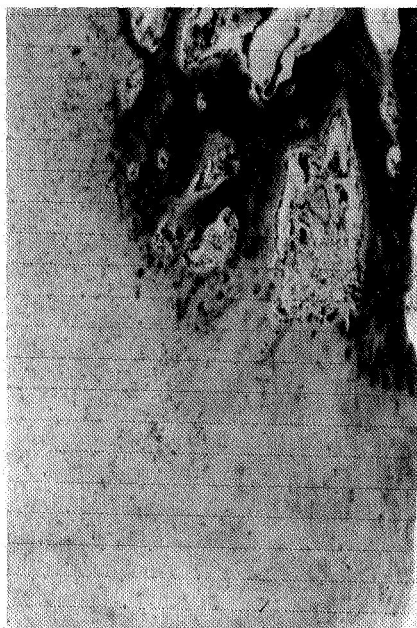


FIGURE 142. Photomicrograph of osification area in the tendo Achilles of a 39-year-old woman. Tendon fibers and new fibrous tissue (top), new fibrocartilage-like cells (center), bone (bottom). HEA stain.

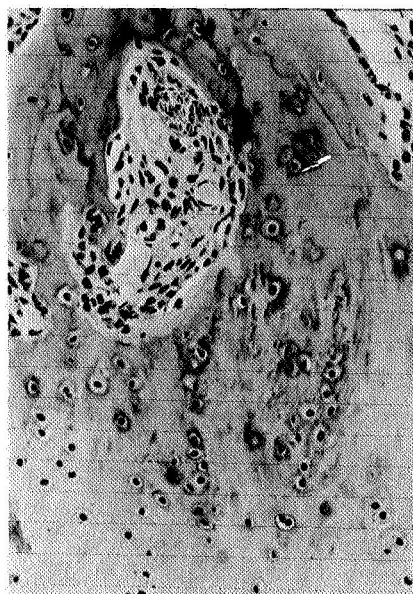


FIGURE 143. Photomicrograph showing the composition of the tissue at the line of division between tendon and bone.

NICHOLS: The question that I originally asked, which seems to have precipitated this discussion, was whether proliferation has to be actively going on at the time that the inductive act occurs? There are two possibilities: cells already formed could be "informed" to suddenly start functioning in a new way; the act of induction might only occur in tissue which was in the act of growing, that is, tissue which was ready to be plasticized. Which is it?

URIST: Dr. Holtzer, can you answer that question?

HOLTZER: There is no simple answer to your question. In part, the reason for this is the difficulty in agreeing on what induction is. The appearance of a bone cell in the developing embryo does not involve one inductive event but many, and the nature of these inductive events probably differs (ref. 210). On a molecular level, we know absolutely nothing about inductive mechanisms in metazoans. Some inductive systems may involve proteins, others lipids, others inorganic ions, others carbohydrates, and so forth.

NICHOLS: Dr. Holtzer, as I understand the "induction" we are talk-

ing about now, it is the creation of a new kind of tissue; I am asking about induction in this sense rather than in the sense you are using.

HOLTZER: That is a wonderful question. Can an exogenous molecule tell a cell that was going to make albumin to make myosin? A quick reading of the embryologic literature might, in fact, leave that impression. By appropriately manipulating bits of embryonic tissues you can demonstrate that here is a group of cells whose descendants would have formed liver cells if left *in situ*. Now, if that bit of tissue is grafted elsewhere, it is possible that the progeny of the grafted cells might differentiate into muscle. But it is worth stressing that there was no single event that can be identified as transforming presumptive liver cells into muscle cells. On the other hand, we know that there was a great deal of mitotic activity in this type of experiment.

NICHOLS: All right. But do you have to have a set of cells in your system that is already mitosing in order to be able to induce them? Or do you start with a resting cell? This is what I am trying to get at.

HOLTZER: In the induction systems I have studied, there is invariably a great deal of mitotic activity. In fact, I know of no well-studied induction system in which the cells are not replicating. Recently, we have proposed that cells might be more responsive to inductive influences immediately following mitosis. For example, we have shown that a presumptive muscle cell does not synthesize myosin until a minimum of 3 to 5 hours after a given mitosis (refs. 124 and 125). Now, for all we know, the parent cell was programmed to produce daughter cells, one or both of which would synthesize myosin. Nevertheless, that particular cell division was necessary to commit the cell to making contractile proteins. Some time ago, we demonstrated that blocking mitotic activity during the inductive interaction between spinal cord and somites could, in fact, inhibit the induction of vertebral cartilage. I believe many experiments in the literature on the effects of nucleic acid inhibitors are due to their blocking cell division and not their action on RNA synthesis.<sup>5</sup>

Indeed, I would go much further and say that what in the past have been referred to as the stepwise events responsible for cell differentiation in the embryo are in large measure changes associated with cell divisions.

URIST: Let us leave the question of whether mitotic activity is essential for induction, and ask if it is possible to apply the hypothesis of Jacob and Monod (ref. 211) to the problem of planning some good new experiments on bone induction.

FREMONT-SMITH: Can you state the hypothesis briefly for ignorant ones like myself?

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<sup>5</sup> Matheson and Holtzer (unpublished observations).



URIST: The Jacob and Monod hypothesis states that the synthesis of proteins by a living organism follows a double genetic control: structural genes determine the molecular organization of the proteins; regulator and operator genes control the rate of protein synthesis through the intermediacy of cytoplasmic components or repressors. The repressors can be either inactivated (induction) or activated (repression) by certain specific metabolites. The system of regulation appears to operate directly at the level of synthesis of the gene of a short-lived intermediate, or messenger, which becomes associated with the ribosomes where protein synthesis takes place. These highly imaginative concepts were developed from a series of experiments on the synthesis of the  $\beta$ -galactosidase and other proteins in cultures of *Escherichia coli*. Watson (ref. 212) and other adventurous young men can be expected to attempt the application of the Jacob and Monod hypothesis to the problem of cellular differentiation and induction systems. Before it is possible to try to use the hypothesis to interpret old experiments and plan new ones on bone induction, it is advisable to define the term "induction." The definition of Jacobson (ref. 213) expresses the view of the most modern embryologists as an "interaction between one tissue [the inductor] and another responding tissue, as a result of which the responding tissue takes a course of differentiation it would not have followed had the interaction not occurred."

The appearance of the bone induction system depends upon the genetic machinery of the species. Bone induction appears only in bony vertebrates. It does not appear in the hagfish, a vertebrate that produces only connective tissue and uncalcified cartilage. It does not appear in the shark, a vertebrate that produces teeth and calcified cartilage but no bone in the skeleton. Only bony vertebrates possess connective-tissue cells with the genetic machinery to be induced to differentiate into bone cells. Later, I will demonstrate an induction system we described in an implant of decalcified bone matrix. If the Jacob and Monod (ref. 211) hypothesis were applicable to this system, it would indicate that the inducer (one or a combination of a number of factors) diffused through the cell to extend as far as the regulator gene.

HOLTZER: You really do not have to go that far, do you?

URIST: You are correct; it is not necessary for the inductor to diffuse as far into the cell as the genetic machinery, but it is also correct that it could, through the intermediacy of a cytoplasmic component or repressor.

HOLTZER: Nobody has ever talked about its hitting the gene. Why do we not leave it in the cell?

URIST: There are at least three possible targets for the inducer: the gene, the ribosomal elements, and the cell wall or plasma membrane.

SAXEN: And in all those sites it could inhibit or stimulate. So there are at least six possible targets.

MCLEAN: It is getting late and since I am now in the chair, I will adjourn this session.

## GENERAL SESSION II

### Discussion Leaders:

DR. MARSHALL R. URIST

DR. FRANKLIN C. MCLEAN

URIST: I am going to ask Dr. Saxén to show some material which does not deal with induction, but before that I would like to ask Dr. Pritchard to make some concluding remarks on the session, "Cellular Differentiation in Bone."

PRITCHARD: Our discussion has centered around the system of cells which lies close to bony surfaces, the cells which are primarily responsible for the formation and resorption of bone matrix and for the production of osteocytes.

The system includes the classic osteoblast and osteoclast and a more generalized type of cell, which used to be called an osteogenic cell or a preosteoblast, but which is now generally referred to as an osteoprogenitor cell. This cell, which is spindle-shaped but otherwise nondescript, was long suspected and recently confirmed to be of the greatest importance in the life of bone. The osteoprogenitor cells are the mother cells which divide to maintain or to increase the population and which differentiate, in suitable circumstances, into osteoblasts, osteoclasts, chondroblasts, and probably fibroblasts. It seems very probable that the reticular cells of bone marrow are really osteoprogenitor cells.

There is some evidence that osteoclasts, and those osteoblasts which do not become osteocytes, may revert to osteoprogenitor cells after their bone-forming and bone-resorbing work is done. This seems to occur in some animals after large doses of parathyroid hormone and in subacute scurvy. On the other hand, there is also evidence that osteoblasts may change to a flat, inactive form—resting osteoblasts—which are later aroused to full functional activity without going through an osteoprogenitor stage. At present, it is difficult to decide whether the flat cells on inactive bone surfaces should be called resting osteoblasts or resting osteoprogenitor cells. It is not known whether osteoclasts may persist in a resting state. In some situations, cells that

are intermediate in position and appearance between osteoprogenitor cells and osteoblasts may be seen. These perhaps could be legitimately called preosteoblasts.

Chemical and histochemical studies *in vivo* and *in vitro* have greatly increased our understanding of the functional activities of the cells mentioned, as electron-microscope studies have greatly clarified our understanding of fine structure. Moreover, we have gone a long way toward correlating structure and function as a result of parallel morphologic and chemical studies.

The big questions which remain concern the factors governing the activity of the population, which control the mitotic activity of the osteoprogenitor cells and which direct their differentiation into active osteoblasts, chondroblasts, and osteoclasts. Many factors are known which are capable of stimulating changes in the appearance and activity of the population—trauma, hormones, nutritional disturbances, mechanical and electrical changes, and so on—but their precise modes of action remain to be discovered. Some of these factors seem to stimulate mitotic activity in progenitor cells; others seem to determine the course of differentiation; and still others may direct the functional activity of the differentiated cells.

In the normal skeleton, the bone-cell system varies greatly in its activity in different places and at different stages of development. Nevertheless, from the time of its inception in the embryo, the system remains throughout the life cycle of an organism either in an active or a resting state. However, bone-cell (and cartilage-cell) systems can arise spontaneously or, following experimental procedures, can be produced in connective tissues which do not normally ossify or chondrify. This is of great theoretic importance, for it implies that a connective-tissue cell population has received new instructions which supersede those they normally receive in development. This brings us into the realm of DNA control by the extracellular environment and the Jacob and Monod (ref. 211) theoretic systems.

This kind of change in cell behavior is called induction, and the factors which bring about the change are called inducing agents or, simply, inductors. As yet, we do not know what these inducing agents are, where they come from, or how they work. It is suspected that, in an inductive situation, one set of cells instructs the DNA of another set of cells—but it is by no means ruled out that some product of cell or matrix breakdown might produce induction, or even that the stimulus is a physical one.

There is one debate of special importance which concerns whether the cells are induced to change their way of life by a single “trigger” stimulus, or whether there is a series of continuing stimuli. Then, again, once a cell has been induced, does it stay induced indefinitely

throughout its own life and the life of its descendants? Or will it revert to its original state as soon as the inducing stimulus ceases? The extreme view is that all cells maintain their form and behavior only so long as they are being stimulated from outside to do so. The classic view, derived from experimental embryology, is that induction is permanent. It may well be that each cell type must be investigated on its own merits in these respects.

Finally, there was a lengthy argument about the relationship of mitosis to induction. On the one hand, it was suggested that only cells recently "born" are capable of responding to inductive agents; on the other hand, it was argued that certain cells might change their mode of life long after the mitosis which gave them birth. Of course, it is conceivable that a cell might receive instructions to become a bone cell, but not respond to those instructions for several weeks, behaving meanwhile like a perfectly ordinary-looking fibroblast; but is this what we usually think of as "induction"? In short, there is an urgent need to define what we mean by induction.

URIST: Thank you very much. Dr. Fremont-Smith has a few comments to make.

FREMONT-SMITH: I made a remark yesterday that cellular differentiation involved the inheritance of acquired characteristics and then I stopped at that point, and this needs to be elaborated.

I merely want to point out that the genes provide the potentials; the environment determines which potentials are going to be realized, and the environment is always present. When a cell is differentiated, it is differentiated as a result of environmental interaction with the gene potentials, and if the cell then gives rise to donor cells of the same specified type, this is, in fact, the inheritance of characters that the cells achieved through the environment. If enough were known about the environment and if it were appropriately modified, the manifestation would also be modified. There is no genic determination which does not involve a crucial aspect of the environment, and if that crucial aspect of the environment is modified, you no longer get the genic determination.

Lastly, I would give two examples. One is the loss of genes from the environment by radiation. This can lead to mutation, which is then inherited. This is an inheritance of a lost character, and the loss came about as a result of environmental influence. Transduction is the opposite—the achievement of new genes coming from a virus—and these new genes then give rise to a mutation, and this mutation breeds true to life; this, then, is the inheritance of a characteristic which came from the environment.

So, although people do not like me to say this, they usually do not deny the facts on which it is based. They wish I would use some other

words. I am not trying to support Lysenko's data, which I think were no good, but what I am saying is that the idea, the dogma, that we are now getting—that we have genetically determined characters—has to be modified to include the fact that there are no genetically determined characters which are not equally determined by the appropriate environment. I wish we had more time to discuss this, but there just is not enough time at this session.

SAXÉN: Dr. Urist said that I am not going to speak any more of the induction problem, but since we have repeatedly discussed the possibilities, and especially the limitations, of tissue-culture methods in studying bone development, it might be of interest to see a few illustrations.

We have recently tested the possibilities of analyzing the action of tetracyclines on calcification *in vitro*. Nevertheless, before any such quantitative determinations can be made, several factors need to be analyzed, and I will briefly mention some of them.

The first factor to exercise a major influence on the results is the age of the donor embryo. When two criteria were used, the length of the mineralized zone, and the total calcium of individual bones, the findings clearly indicated an optimal age of 17 days. Bones from embryos younger than this did, in fact, develop and calcify, but at a rate slower than those of the 17-day embryos, and when the rudiments were removed from 18- to 19-day embryos, the growth rate slowed down once more. These results thus indicate the importance of a homogeneous starting material in such experiments *in vitro*. The great influence exerted by age is clearly discernible in figure 144, which illustrates the incorporation of radiocalcium into bones of 16- and 17-day embryos, respectively.

In our different experiments, the tissue-culture medium had to be changed frequently (incorporation experiments, transfer to and from the tetracycline-containing medium, etc.). This led to the effect of such repeated changes being tested with a somewhat unexpected result. Seemingly, one change during the 10- to 12-day cultivation period did not affect the growth and calcification, but if the change was repeated once after two days, the calcium uptake was definitely diminished. The explanation of this phenomenon might be found in a certain micromilieu provided by the tissue cultivated for some time and removed by repeated washings. In practice, this means that unnecessary changes of the medium should be avoided and, when a change has to be made, a similar manipulation should be carried out for the control cultures.

The next thing to be checked was the "aging" of the medium if changes were avoided. An experiment was made, in which bone rudiments were cultivated in a medium for 14 days, measured, and dis-

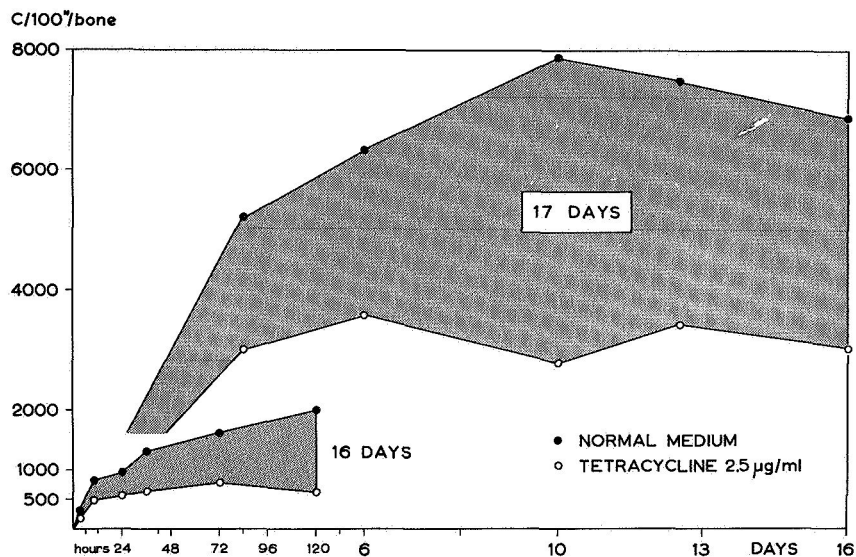


FIGURE 144. Incorporation of radiocalcium into bones of 16- and 17-day embryos.

carded. Subsequently, the same medium was reused for a similar culture, and the growth of the bones was again studied. The results clearly indicated that, at least during this 28-day period of total cultivation time, the same medium supported good growth, elongation, and calcification.

So much for the technical difficulties and limitations. If I may, I will very briefly go through some of the results, indicating what kinds of information we can and cannot expect from tissue-culture studies.

RAISZ: I think this is fascinating, and it seems astounding to me. I wonder if you could tell us what the volume of medium was, relative to the amount of culture material.

SAXÉN: It was 1 to 100.

RAISZ: That is the usual tissue-culture ratio.

SAXÉN: Yes. Then, of course, we had to determine the total calcium and ionic calcium in the medium, and the changes are very slight.

One of the things for which our tissue-culture conditions seemed to be suitable was study of the incorporation of tetracycline into the bones. Frozen sections, made after different lapses of time, and studied in UV light showed a very rapid incorporation of the drug into the rudiments; even after some 30 minutes, clear fluorescence was visible. Later, this incorporation was determined by the employment of labeled tetracycline. Figure 145 shows the total incorporation

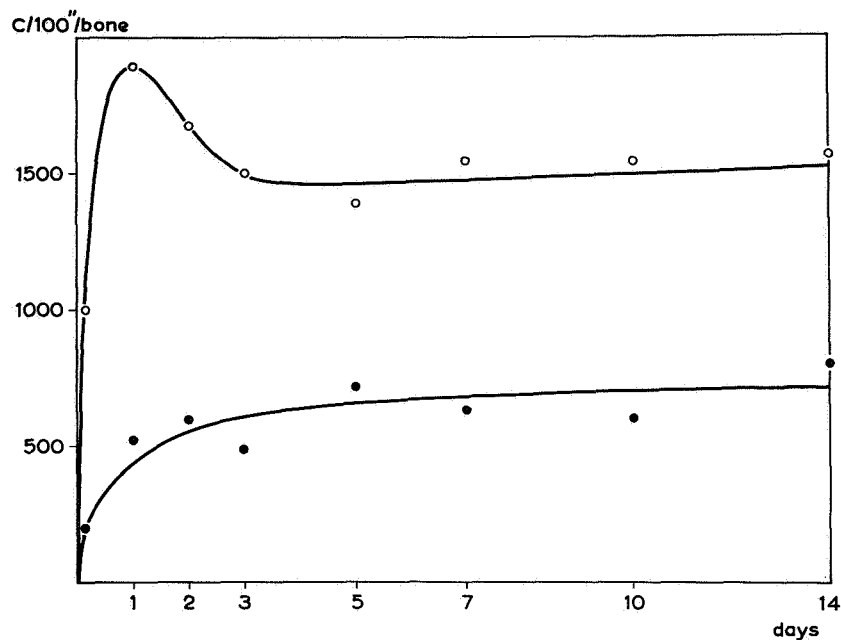


FIGURE 145. Incorporation of tetracycline into embryonic bone rudiments.

as a function of time. The upper line indicates the content of tetracycline in the bones immediately after treatment, and the lower line the content after 24 hours' subsequent cultivation in a cold medium. The results indicate that approximately four-fifths of the incorporated tetracycline leaks out rapidly, whereas one-fifth is retained in the bones. Figure 146 shows this leakage in more detail, and suggests that the portion of tetracycline retained in the bones after the first 24 hours will remain in the bones without any major changes.

BAUER: Which method did you use for measuring the amount of tetracycline?

SAXÉN: Tritiated tetracycline was counted in a liquid scintillation spectrometer after short-term treatment of the bones with hyamine.

Figure 147 illustrates the dependence of concentration on the incorporation of radiocalcium. It seems that a concentration of  $0.1 \mu\text{g/ml}$  of tetracycline does not affect the uptake of calcium during this period of cultivation, whereas a definite diminution was noted at a concentration of  $1.0 \mu\text{g/ml}$  and  $10.0 \mu\text{g/ml}$ .

HOLTZER: After the tetracycline, do you think your system is still living? I was not quite sure I followed that.

SAXÉN: You mean whether the bones are living? Yes, they are.



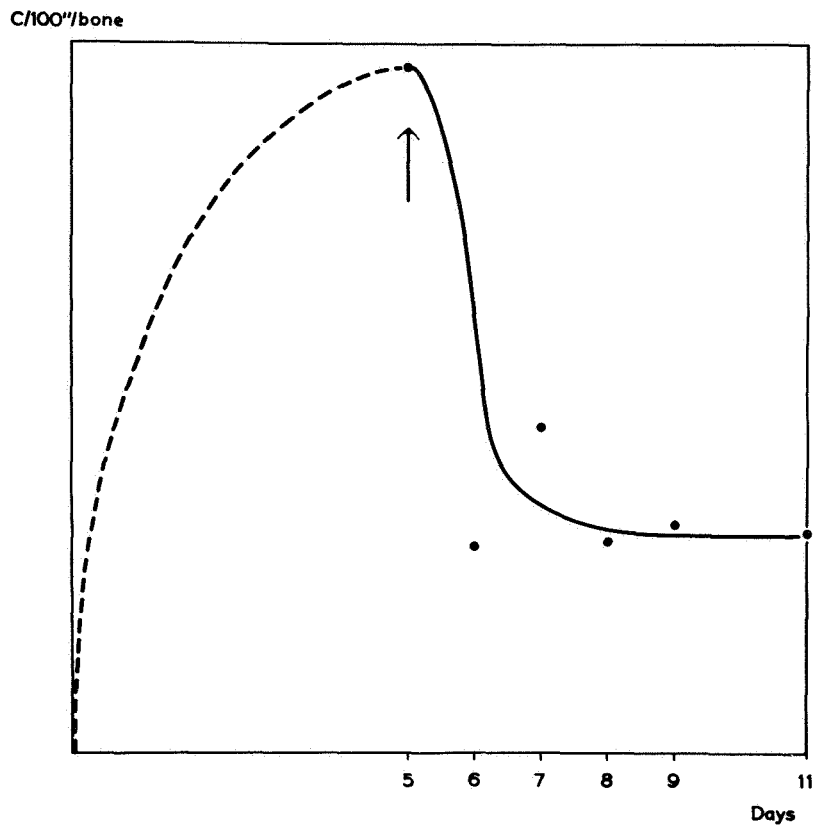


FIGURE 146. Incorporation of tetracycline into embryonic bone rudiments as a function of time.

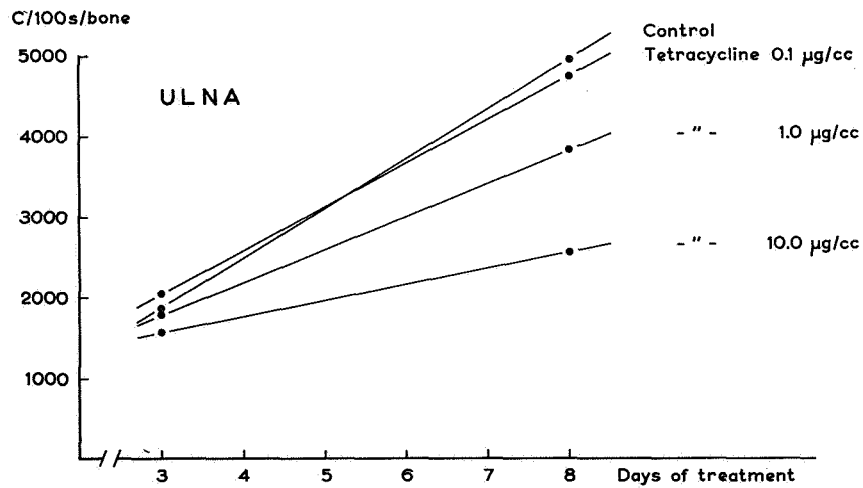


FIGURE 147. Dependence of tetracycline concentration on the incorporation of radio-calcium in embryonic ulna.

HOLTZER: And you can show this by putting them back in labeled calcium which they take up?

SAXÉN: Yes. I will come to that soon. The actual experiment which demonstrates the viability of the bones was performed by application of the thymidine leakage method. Here, the rudiments were labeled with  $^3\text{H}$ -thymidine prior to tetracycline treatment, and later the amount of label in the culture medium was studied. No differences were noted between the control cultures and the cultures cultivated in the presence of tetracycline up to 100  $\mu\text{g/ml}$ .

HOLTZER: Can you say something about the place in the tissue? Is this definitely an extracellular location which is not affecting cell viability on the part of tetracycline? It would be very nice if you could.

SAXÉN: At the moment, we do not know much about the mechanism, but we are investigating that in another system—a continuous cell line. With respect to the bone cells, I really believe that they are still alive after several days of tetracycline treatment. This is observable from experiments in which the bones have been cultivated in the presence of tetracycline for some days and then transferred to normal medium. Within a day or two, a recovery is achieved, and the bones incorporate radiocalcium at a normal rate. Not until 5 to 6 days of cultivation does the effect seem irreversible.

Even here we have good evidence that the cells survived. They are alive, but there is no recovery from the sixth day onward.

This summarizes my concluding remarks. I merely wanted to show one example of how organotypic culture methods can be used in studies of hard tissues, particularly bone.

URIST: Would you like to speculate on the site of action of the tetracycline in the cell?

SAXÉN: How it acts?

FREMONT-SMITH: How and where; both.

SAXÉN: Many people have speculated on that. One possibility, of course, is that it seems to interfere with the deposition of calcium into the crystals. At present, we are interested in knowing whether it interferes with collagen synthesis, and we are going to use proline incorporation and electron-microscopy studies to do that. As yet, I have no conclusive data. That is why I was very much interested, Dr. Talmage, in your similar study with salicylates—where you get an inhibition of the collagen.

BAUER: In your article in *Science* (ref. 214), you made the point that you found the effect on collagen synthesis by tetracycline concentrations approximately the same as those encountered after tetracyclines have been administered for therapeutic reasons.

SAXÉN: The therapeutic concentration during a normal treatment is on the order of 4 to 5  $\mu\text{g/ml}$ .

BAUER: Dr. Urist has reviewed evidence that tooth development has been found to be impaired in infants from mothers who have received tetracycline therapy during pregnancy.

URIST: We were very interested in the subject, because the mothers that had been treated with tetracycline during pregnancy for lung infection, and infants treated for various infections, presented a very disturbing problem. When the teeth of the child erupt, they are bright yellow and then turn brown; as you can imagine, it is a very disturbing sight to the mother. We performed experiments on rabbits and concluded that the tetracycline was bound to the crystal surface of apatite. After exposure of the teeth to sunlight, some tetracyclines are unstable, degraded, and turn from yellow to brown. Chlortetracycline is more stable than tetracycline phosphate (ref. 215).

HOLTZER: Why is it used in the clinic?

NICHOLS: It is an antibiotic.

BAUER: In tissue cultures, one usually studies effects of agents in very much higher concentrations than those normally encountered in real life conditions. This is why it is so highly interesting to find that effects of tetracyclines on mineralized tissues apparently occur *in vitro* and *in vivo* at approximately the same concentrations of this agent.

SAXÉN: If we can make any conclusions of the action *in vivo*, the concentration is of this very same order.

PECK: One of the problems associated with clinical use of tetracycline is that outdated or old tetracycline undergoes chemical changes which render it toxic to humans, particularly with respect to renal problems. I wonder if you have any evidence that the tetracyclines are not undergoing these changes in your culture medium.

SAXÉN: It must be a very rapid change, because we get the effect in 2 days with a fresh, new, and purified tetracycline preparation.

PECK: I do not know how it would behave under different circumstances. I do not think there is any evidence one way or the other.

NICHOLS: Dr. Holtzer, your question is one which you should never ask a clinician. There are many people who think these drugs are used far too indiscriminately in the practice of medicine. Tetracyclines are very popular because they have a broad spectrum of activity against a wide variety of organisms.

SAXÉN: It seems to be very popular for treatment of chronic urinary-tract infections during pregnancy, which I have been told are rather tricky.

NICHOLS: There are complications with the use of these drugs. While many people tolerate them quite well for long periods, secondary infections by nonsensitive organisms often develop during their use.

RAISZ: Dr. Saxén's very beautiful studies have brought up two points. One is the problem of dose, and I would like to rise to the challenge that Dr. Bauer has brought up on this score. In tissue culture, exogenous drugs can have effects in doses similar to those which act *in vivo*, unless there are specific carrier or conversion systems for the drug. Therefore, I do not think that we can say that tissue-culture systems always respond to concentrations higher than those of the blood. Dr. Peck has been able to get responses with ascorbic acid at concentrations which are close to those found in cells. I think many other tissue systems have been refined in tissue culture to the point where they show responses to a variety of agents at concentrations near those found *in vivo*. In this regard, I should like to show some data which indicate that parathyroid hormone in normal rat blood apparently affects the radiocalcium release from embryonic bone in tissue culture (table XXXIV).

These were made on heat-inactivated sera from normal rats and thyroparathyroidectomized rats. Calcium release measured in a medium containing normal rat serum is relatively high, and this value is lowered by the addition of serum from a guinea pig immunized with purified bovine parathyroid hormone. If one uses serum from thyroparathyroidectomized rats, there is a much smaller release of calcium, probably largely due to physical chemical exchange of the surface radioactivity with the medium, and there is no inhibition of this by antibody. A dose of 1  $\mu\text{g}/\text{ml}$  of purified bovine parathyroid hormone added to serum from thyroparathyroidectomized rats caused a response

TABLE XXXIV  
EVIDENCE FOR DETECTION OF PARATHYROID HORMONE IN NORMAL RAT SERUM BY  
BIOASSAY

Added serum (5 percent)	<sup>45</sup> Ca release, cpm/0.1 ml of medium		
	Normal serum	TPTX serum	TPTX serum and PTH
Normal guinea-pig serum.....	2190 $\pm$ 310	<sup>a</sup> 1010 $\pm$ 150	2110 $\pm$ 150
Anti-PTH guinea-pig serum.....	<sup>a</sup> 1020 $\pm$ 150	<sup>a</sup> 1040 $\pm$ 170	<sup>a</sup> 1000 $\pm$ 20

NOTE.— Values are means  $\pm$  S.E. of 4 bones.

<sup>a</sup> Significantly different from values in normal rat serum or TPTX serum plus PTH,  $p < 0.02$ . Data are for <sup>45</sup>Ca release from bone cultures in serum from normal or thyroparathyroidectomized (TPTX) rats. Effect on <sup>45</sup>Ca release of normal serum, or when bovine parathyroid hormone (PTH, 1  $\mu\text{g}/\text{ml}$ ) is added to serum from TPTX rats, can be abolished by adding serum from a guinea pig immunized with bovine PTH.

similar to that obtained in normal serum and this was also inhibited by antibody.

I believe that this type of data indicates that if we can overcome the problems of species difference and some of the difficulties in maintaining tissue cultures, we should expect to achieve responses to normal blood levels of hormones and to the usual *in vitro* doses of drugs. In other words, we should not accept the requirement of a high concentration *in vitro*, but should work toward showing effects with lower concentrations. One other point that I would like to make is that Dr. Saxén's findings are contrary to the usual dogma of tissue culture, which is that the medium must be changed frequently. I would like to hear his comment on this, because this dogma has been religiously adhered to and, of course, we could save a lot of work if Dr. Saxén would promulgate this new doctrine.

SAXÉN: It is very difficult to comment on that, because it is just a finding. I was surprised myself, and I have repeated the experiment. It may be that the metabolism of the bone is low—I do not know. Another thing which I tried to show is that in certain experiments the medium should not be changed, because a different effect is obtained.

NICHOLS: Dr. Saxén, one wonders immediately whether there is something accumulating in the incubation medium which is, so to speak, "good" for the bone. Have you done the critical experiment of taking the medium off the culture and then replacing it again? One wonders whether the actual maneuver of removal is important or whether it is the medium itself?

SAXÉN: I have not done this, yet I feel that it is the maneuver because in some instances we have to be very careful when we measure them daily. If the tissues are just shaken, decreased growth results.

HOLTZER: May I suggest that really the problem you are alluding to, at least as I can understand it, is that changing the medium promotes mitosis. Does a change in medium promote anything else? I am not aware of it. It might very well, but I know that when we have had occasion to measure, mitosis has always occurred. My bet would be that whatever the constant situation is after 6 or 7 days (and you say even changing the medium essentially limits it), mitosis is discouraged, and, conversely, in an obligatory way, the uptake of calcium is promoted, and you have an inverse ratio, whatever the requirements are for reproduction. This is the negative aspect in tissue culture.

SAXÉN: I do not want to go back to the environment-induction problem, but as you know, embryologists have repeatedly stressed the microenvironment, which cells and tissues create as they grow in tissue culture, so it may well be that you just wash it out.

NICHOLS: Yes, indeed. The reason I was intrigued was that I have found that the packing of the cells in bone-cell suspensions seems to be

a very critical factor. In my system, for instance, the oxygen uptake of the cell population per milligram of DNA increases as the concentration of cells in the medium increases, which is absolutely upside down from what one would expect if diffusion were a limiting factor.

BUDY: In defense of *in vitro* systems, I would like to point out that André (ref. 216) did a gross distribution study of tritiated tetracycline, and the distribution within bone certainly paralleled what you have shown so beautifully in your tissue-culture system.

SAXÉN: I may say that this is not the only report.

BUDY: There were several others, but André's thesis was——

SAXÉN: He was the first; others have made use of that typical fluorescence.

BUDY: There is a very close relationship between André's results and what you have shown. I am speaking in reference to gross autoradiography.

SAXÉN: I guess this may take care of one-third of the tetracycline. Where the rest of it is I do not know. We do know that dying cells do take up tetracycline, and that peak at the very beginning of any tissue culture may very well mean that there are dying cells taking up tetracycline; this fits with our observations of the thymidine leakage. There is a similar peak in the thymidine concentration of the medium at this time.

URIST: Dr. Arnaud, have you some material you would like to present?

ARNAUD: Yes, I think so. I should like to expand some on the comments I have made during the discussion and offer support for them with recently obtained data. First, the matter of using parathyroid extract in *in vitro* systems. When fractionating crude parathyroid extracts on Sephadex G-100 columns, as in the routine purification of parathyroid hormone, one obtains a series of protein peaks which have neither calcium mobilizing nor phosphaturic activities in the parathyroidectomized rat (ref. 217). Hawker and Glass have purified two of these and have characterized them chemically. They bear no relationship to the classic parathyroid hormone in this regard either. Nevertheless, they have interesting properties in *in vitro* systems. Dr. Tenenhouse has shown that one of them, peak 3, stimulates glycolysis when added *in vitro* to ascites tumor cells, an effect reminiscent of that which has been reported when parathyroid extract has been added *in vitro* to ascites tumor cells, and also reminiscent of that which has been reported when parathyroid extract has been added *in vitro* to incubated bone chips. The other polypeptide, peak 2, has been studied extensively in mitochondrial systems by Rasmussen and Ogata (ref. 218). Superficially, its effects resemble those of parathyroid hormone in that it stimulates mitochondrial phosphate accumulation.

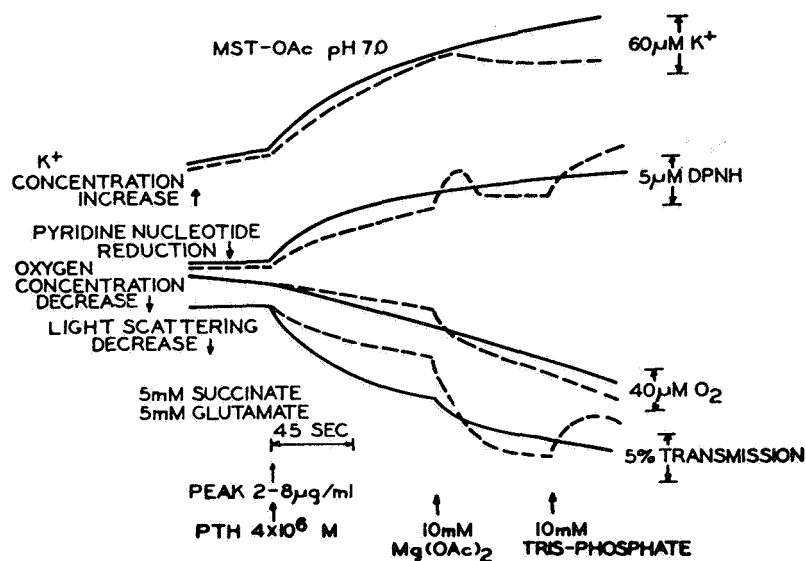


FIGURE 148. Responses of isolated rat liver mitochondria to successive additions of 10 millimoles  $\text{Mg}(\text{OAc})_2$  and 10 millimoles  $\text{Tris-phosphate}$  in the presence of  $8 \mu\text{g/ml}$  of peptide, peak 2 (—) compared with the responses seen in the presence of  $4 \times 10^{-6} \text{ M}$  parathyroid hormone (----) in a mannitol-sucrose-Tris-acetate medium. [From ref. 218; reprinted by permission of the publisher.]

However, using more discriminating methods, it is easy to show that the effects of these polypeptides on mitochondrial metabolism are quite different. Figure 148 shows the effects on the oxidation of pyridine nucleotide, oxygen consumption, and light scattering (mitochondrial swelling) of rat-liver mitochondria incubated in a mannitol-sucrose-acetate medium at pH 7. Parathyroid hormone causes only small changes in these parameters until either magnesium or potassium is added. At this time, there is rapid pyridine nucleotide oxidation, increase in oxygen consumption, and mitochondrial swelling. Partial reversal of swelling is effected by the addition of phosphate. In contrast, the addition of peak 2 is followed immediately by irreversible changes in all indices. Neither magnesium nor potassium is required for these effects.

I think that we can assume that both of these peptides (peaks 2 and 3) are present in parathyroid extracts which are commercially available at this time. The use of the extract in studies *in vivo* is probably safer than studies *in vitro*, but I personally use great caution when interpreting any work done with material other than the purified preparation.

Now we will shift to another topic. To employ physiologic techniques in some of our biochemical studies, we found it necessary to

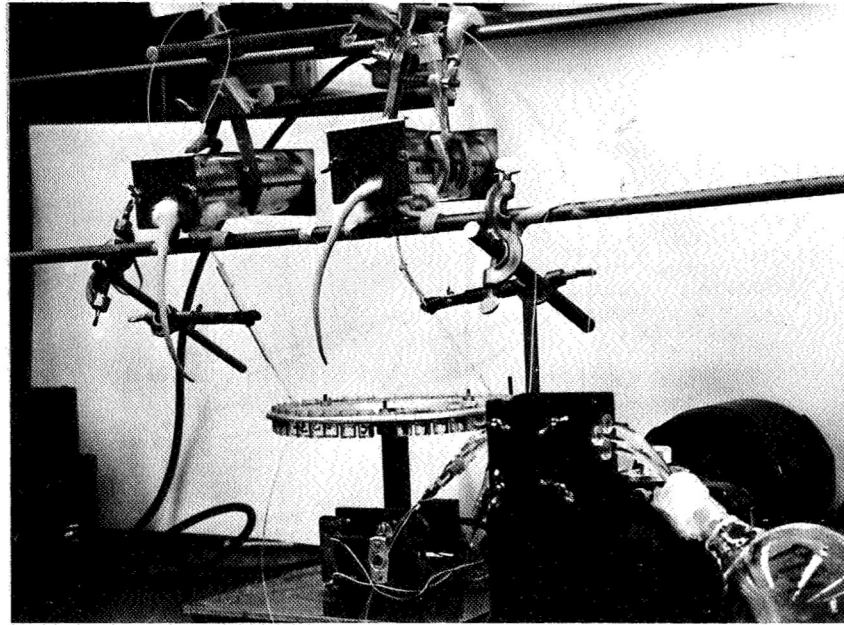


FIGURE 149. Two thyroparathyroidectomized rats set up for the long-term perfusion of parathyroid hormone.

develop a relatively precise technique for the study of the effect of hormones *in vivo* (ref. 54). Figure 149 is a picture of two rats that, believe it or not, are relatively comfortable. They are either parathyroidectomized or thyroparathyroidectomized and can be maintained for up to 4 days by perfusing them at a constant rate with an electrolyte solution which contains calcium, sodium, potassium, magnesium, and glucose via a polyethylene catheter inserted into the external jugular vein. Another polyethylene tube is placed in the urinary bladder to collect urine. A fraction collector is used and urine samples of any time interval can be obtained so that a semicontinuous monitoring of urinary electrolyte composition is easily accomplished. Also, blood can be obtained for analysis at judicious times during an experiment by slashing the animal's foot with a sharp razor blade. Animals are perfused at rates of 3 to 4 milliliters per hour, and there is no significant water retention. These techniques are modifications of the procedures described by Cotlove (ref. 219) and by Pechet.<sup>1</sup>

The work I would like to show you now was done with Dr. Anast during the year he spent in our laboratory on leave from the Department of Pediatrics at the University of Missouri School of Medicine

<sup>1</sup> Pechet, M. Personal communication.



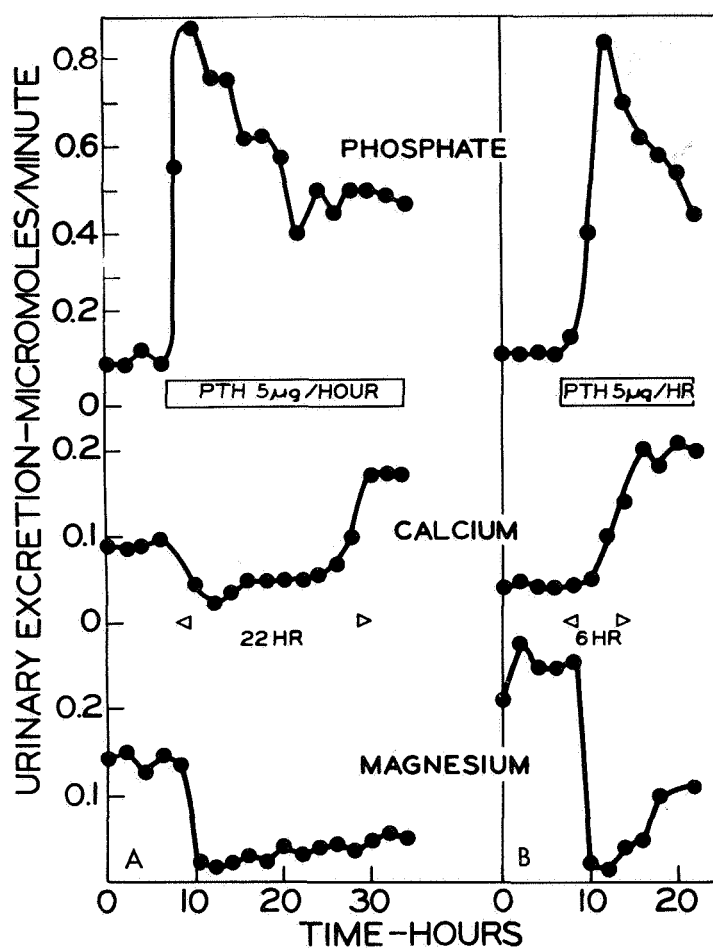


FIGURE 150. The rate of excretion ( $\mu$ moles/min) of phosphate, calcium, and magnesium in the urine of parathyroidectomized (a) and thyroparathyroidectomized rats (b) before and during the constant infusion of parathyroid hormone at a rate of 5  $\mu$ g/hr. The values represent the means obtained from four rats in each set. [From Anast et al. (ref. 220); reprinted by permission of the publisher.]

(ref. 220). It was designed to demonstrate the importance of thyrocalcitonin in the responses of the rat to an excess of administered parathyroid hormone and employed the rat perfusion system I have just described.

Figure 150 compares the responses of the parathyroidectomized (PTX) rat, on the left, with the thyroparathyroidectomized (TPTX) rat, on the right, to a constant infusion of parathyroid hormone (5  $\mu$ g/hr). There is a rapid and sustained phosphaturia in both animals, but it is

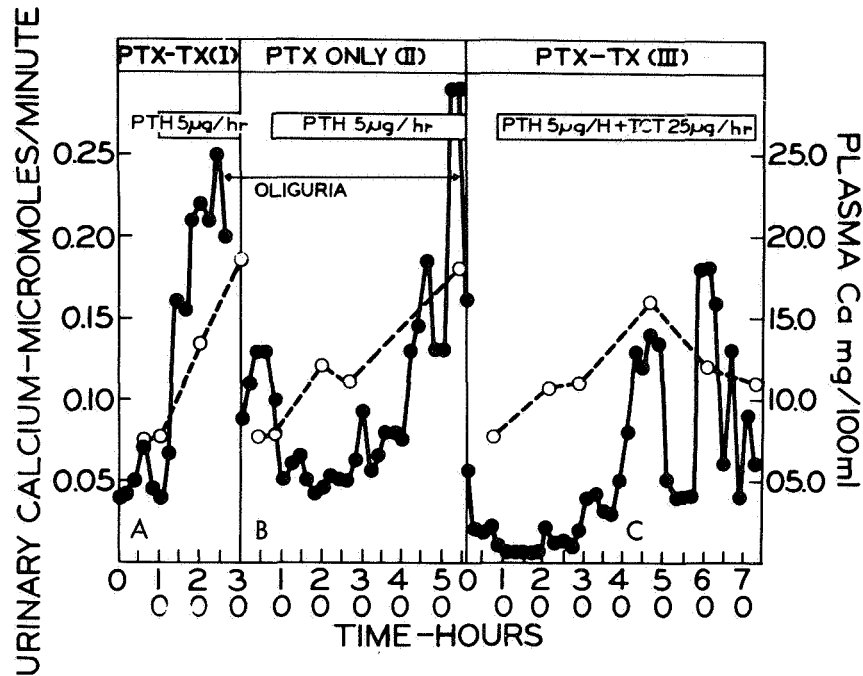


FIGURE 151. Plasma calcium concentration (○- - -○) and rate of urinary calcium excretion (●—●) before and during the infusion of purified parathyroid hormone in (a) a thyroparathyroidectomized rat; (b) a parathyroidectomized rat with an intact thyroid gland; and (c) a thyroparathyroidectomized rat receiving a simultaneous infusion of thyrocalcitonin (25  $\mu$ g/hr). The thin arrow with *oliguria* written under it indicates that oliguria developed after 16 hours of parathyroid hormone infusion in rat A and after 48 hours in rat B. No oliguria was seen in rat C. The values represent the means obtained from four rats in each set. [From Anast et al. (ref. 220); reprinted by permission of the publisher.]

clear that their responses differ with respect to calcium excretion. The animal without thyroid-parathyroid glands develops hypercalciuria within 6 hours and dies within 14 to 16 hours of hypercalcemia and nephrocalcinosis, whereas hypercalciuria is markedly delayed in the animal with a thyroid gland and nephrocalcinosis is rarely observed. It is interesting, with regard to our previous discussion concerning the rapid action of parathyroid hormone, that a detectable increase in the plasma calcium concentration can be observed within one-half hour in the TPTX rat, but not until 2 to 3 hours in the PTX rat. We think that the differences between the responses of these animals is the presence of circulating thyrocalcitonin in one and its absence in the other.

Studies of the influence of the concomitant perfusion of purified thyrocalcitonin on the pattern of response of the TPTX rat to parathyroid hormone support this notion. Figure 151 illustrates this,

and shows clearly the importance of the presence of the thyroid gland when one attempts to interpret the physiologic or pharmacologic effects of parathyroid hormone administration. Either the presence of the thyroid gland (fig. 151(b)) or the perfusion of thyrocalcitonin (fig. 151(c)) dramatically protects against the hypercalcemic and toxic effects of parathyroid hormone observed in the animal without thyroid-parathyroid glands or thyrocalcitonin (fig. 151(a)).

I should like to point out, in my last few remarks, a finding which we have only recently observed (fig. 152). Using the rat perfusion system, we have been able to show that the infusion of thyrocalcitonin into TPTX rats either alone (fig. 152(b)) or with a maintenance dose of 1  $\mu\text{g/hr}$  of parathyroid hormone (fig. 152(a)) induces transient phosphate and sodium excretion, and a sustained decrease in the rate of calcium and magnesium excretion. The important question is whether this is a

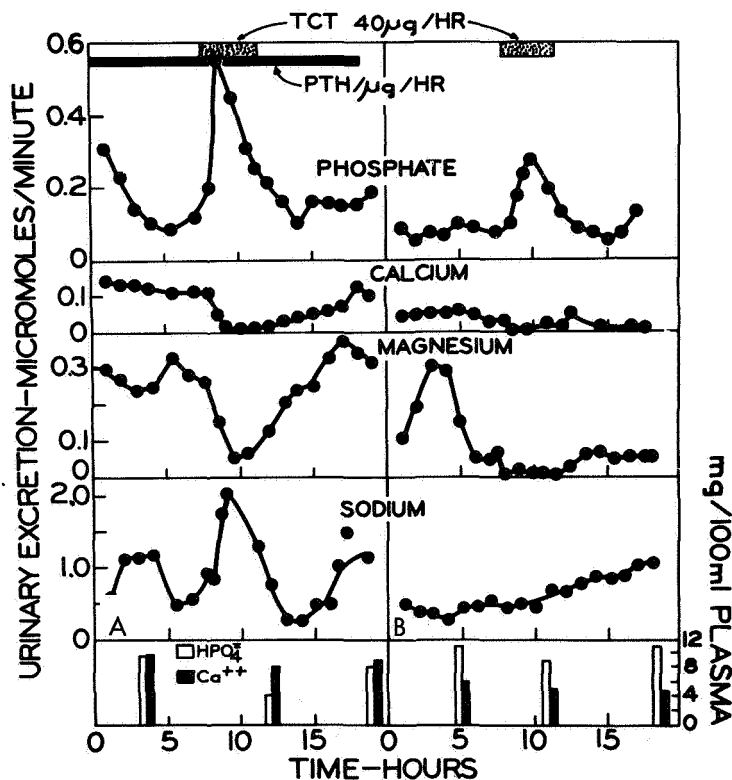


FIGURE 152. The effect of thyrocalcitonin (TCT) infusion upon urinary and plasma electrolytes in thyroparathyroidectomized rats (b) and similar animals maintained on a constant infusion of parathyroid hormone (a). The values represent the means obtained from four rats in each set.

direct effect of thyrocalcitonin or is related to changes in the local ionic environment of the kidney tubule. This is currently under study. Of great interest is the fact that the total phosphate which is excreted during thyrocalcitonin administration corresponds closely to the calculated decrease in the phosphate content of the extracellular fluids. This immediately raises the question as to whether the hypophosphatemic effect of thyrocalcitonin, under physiologic conditions, is due exclusively to its influence on bone.

URIST: Thank you very much. Dr. Copp, have you any comments?

COPP: I believe Munson (ref. 221) also observed an effect on phosphate in the nephrectomized animal.

ARNAUD: One thing, of course, that we do not know very much about is soft-tissue phosphate. As a matter of fact, we do not know a great deal about soft-tissue calcium either, and this is a matter which requires considerable study, as it is extremely difficult to measure soft-tissue calcium accurately.

COPP: Despite blood changes, Kenny and Heiskell (ref. 222) reported no significant changes in intracellular calcium and phosphate following thyrocalcitonin administration to rats.

ARNAUD: It must be remembered that every tissue is perfused by large quantities of extracellular fluid. If there is a decrease in the extracellular fluid calcium, one would also expect a decrease in the cellular calcium, unless careful measurements of the extracellular fluid space of the tissue and its calcium content are taken into consideration.

COPP: There is no indication that hypocalcemia is associated with any significant reduction in intracellular calcium, although extracellular calcium is reduced.

ARNAUD: Yes; but the tissue calcium is measured along with the extracellular fluid calcium. None of the studies done so far has accounted for the extracellular space.

COPP: I assume that it drops with the fall in plasma calcium concentration. It must.

ARNAUD: I am only suggesting that, on the basis of precedent (actions of other hormones), tissues other than bone might be responsive to thyrocalcitonin.

COPP: I think that this is a very important aspect of phosphate metabolism. There must be rapid movement of phosphate in and out of cells with changes in metabolic activity, since phosphate is required for the high-energy phosphate bonds involved in energy transfer.

FREMONT-SMITH: A factor that seems to have been regularly neglected is that probably the tissue that has the most constant calcium content in the body is cerebrospinal fluid. This does not vary, even with marked variations in plasma calcium concentration, whether the plasma

calcium is raised and maintained in the dog for several days at 12 to 14 mg/100 ml, or whether it is decreased by lowering the plasma calcium to the concentration you get after the thyroid has been removed. But the only point at which the spinal-fluid calcium begins to decrease is the point at which the plasma calcium falls below 4.5 or 4.0 mg/100 ml, which is the concentration of ionized calcium or that of the spinal-fluid calcium. In the very few instances which I have seen in the literature, the spinal-fluid calcium concentration then drops down to about 3, right with the plasma calcium.

These are, of course, extremely low concentrations; a comparable result is rarely obtained. Everybody ignores this because they insist that spinal fluid has nothing to do with calcium anyway—"It is a secretion; therefore, let us forget about it." But it is the most constant tissue fluid for calcium in the body, I believe.

ARNAUD: I think the fact that these agents do not affect the membranes which are responsible for the regulation of the spinal-fluid calcium is terribly important in terms of brain homeostasis.

NICHOLS: There are some experimental data on this subject from a couple of laboratories, notably Katzman's. These suggest that spinal-fluid calcium concentration is closely controlled independently of the concentration in the plasma. It looks as if there may be a transport system somewhere in the brain, presumably with its own "calciostat," if one may use that word, which controls cerebrospinal-fluid calcium concentration by transfer of calcium from blood to the cerebrospinal fluid (ref. 223).

FREMONT-SMITH: Does anybody know what happens to the spinal-fluid calcium when a considerable amount of citrate is injected into the blood? I have always thought that this was one way in which it could be determined whether changes in the spinal-fluid calcium occur.

COPP: I can give some observations made by Dr. Shim<sup>2</sup> in our laboratory on the effects of EDTA. When the noncomplexed plasma calcium concentration is reduced as low as 2 mg/100 ml by rapid EDTA infusion, there is no tetany.

FREMONT-SMITH: What happens to the spinal fluid?

COPP: Spinal-fluid changes lag about 3 hours behind plasma.

FREMONT-SMITH: But it does come down?

COPP: It does come down.

FREMONT-SMITH: This lag, by the way, is characteristic of almost everything in spinal fluid.

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<sup>2</sup> S. S. Shim, Department of Physiology, University of British Columbia, Vancouver, Canada. Personal communication.

COPP: Yes; there certainly is such a lag. However, if you lower the spinal-fluid calcium but keep plasma calcium normal, the animal has muscle spasms resembling severe tetany.

FREMONT-SMITH: How do you lower the spinal-fluid plasma?

COPP: By injecting EDTA into the spinal canal. This would suggest that the tetany may be a central rather than a peripheral phenomenon.

URIST: Dr. Arnaud, is total soft-tissue calcium, total extracellular fluid calcium plus intracellular fluid calcium, difficult to measure accurately?

ARNAUD: No. I think there are problems with respect to interfering substances such as phosphate; so much so that when we came to measure calcium in mitochondria, we went to activation analysis and found that even this was somewhat difficult in terms of reproducibility.

URIST: Dr. Heaney, have you made measurements of soft-tissue calcium?

HEANEY: No.

COPP: We measured it with EDTA (ref. 224).

URIST: Phosphate does not interfere with the EDTA method unless there is hyperphosphatemia or large amounts of phosphorus in the tissue.

COPP: We find, with hypocalcemia, you have a slight fall which I have attributed to the fall in extracellular fluid calcium. With calcium perfusion, you have a slight increase, but I think this is accounted for almost entirely by extracellular fluid calcium.

ARNAUD: The point is that it can be done. It is just very difficult to do.

HEANEY: I will second that. It is very hard to do and it is very tedious. There is a great deal more calcium in soft tissue than can possibly be accounted for by the extracellular fluid content. It is not in the cell sap.

COPP: Especially in tissues with a high level of sialic acid or mucopolysaccharides. For example, there is a high calcium concentration in thyroid and salivary glands.

HEANEY: Skin has 150 to 250 milligrams of calcium per kilogram.

HOWELL: Hypertrophic cell cartilage contains twentyfold to thirtyfold per unit of wet-weight calcium measured grossly. We obtained some of our samples from muscle insertion sites, and that is probably fairly close to an extracellular fluid. It has 1 gm/100 ml protein and the calcium measured in that was about 7 mg/100 ml.

PECK: It has been postulated that there is actually an ion cloud which is very rich in calcium that surrounds many cell types, particularly the muscle cells, and if the concentration of calcium in the fluid which

surrounds all cells is measured, it increases dramatically, the closer you get to the cell membrane.

ROWLAND: What mechanism can control this?

PECK: Well, it possibly has to do with the actual composition of the cell membrane itself. There is very little information about this.

URIST: Dr. Peck, have you some material you would like to present?

PECK: Yes. We have been discussing the problem of membranes, and the concept that hormones, inducers, and stimulators act by modifying the function membranes is not a new one. We have been interested in the possibility that hormones can modify the lipid composition of cell membranes, in particular the membranes of our isolated bone cells, and it is only recently that the techniques for isolating and separating lipids have become sophisticated enough to be applied to relatively small amounts of tissue.

Much of the work in designing the techniques for these separations was done by Marinetti in 1962 (ref. 225). I have done these studies in collaboration with Dr. Thomas Dirksen in the Department of Biochemistry (ref. 226). The main question, or the only question, that we have answered to date is, Can the cells synthesize lipid, and if so, what kind of lipid can they synthesize?

Figure 153 shows the time curve of incorporation of  $^{14}\text{C}$ -glycerol into the lipids of cells maintained in culture on a flat surface (ref. 227). Most of the lipid that is formed appears in the cell layer. A very small percentage appears in the supernatant medium that overlies the cell layer.

The interesting thing is that the large percentage of incorporated glycerol appears in phospholipid which, as you all know, constitutes the majority of lipid in cell membranes. A large proportion of the radioactivity is in lecithin or choline phosphoglyceride.

There is an appreciable incorporation of glycerol into neutral lipid and a small but significant incorporation into phosphatidyl ethanolamine and other phospholipids.

I think the only implication is that we have a method for studying the possible mechanism of action of hormones at the level of the cell membranes. It is possible, for example, that a hormone can alter the transport processes of cell membranes by actually altering those factors that modify the lipid composition of the cell membrane. We have recently done some studies on the addition of parathyroid extract—and I must emphasize, in view of Dr. Arnaud's comments, that this is the crudest material—on the ability of these cells to synthesize lipid. We have found some evidence to suggest stimulation of incorporation of glycerol into phospholipid moieties, particularly lecithin and phosphoethanolamine. We have also tried one experiment with a more

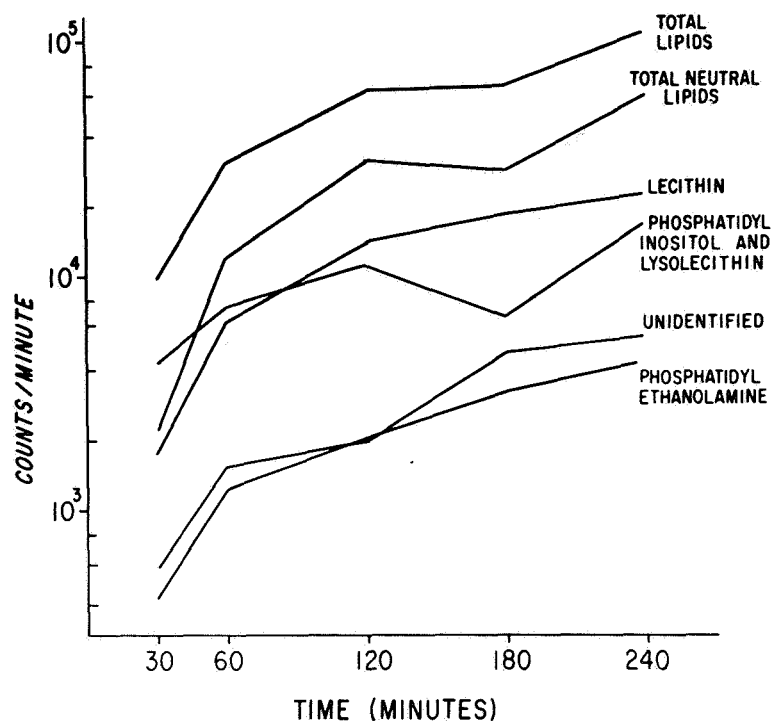


FIGURE 153. Time curve of incorporation of 1,3-<sup>14</sup>C-glycerol into the phospholipids of rat calvaria. After incubation, lipids were extracted into chloroform-methanol and separated by chromatography on silicic acid-impregnated paper. Papers were cut into horizontal strips corresponding to the position of standards chromatographed simultaneously, and each strip was counted in a liquid scintillation spectrometer. [From ref. 227; reprinted by permission of the publisher.]

purified, but not completely purified, preparation, and the data suggest again a possible stimulation of this incorporation. If subsequent studies confirm this, we will direct our attention to isolating various cell fractions to see whether these changes are reflected, for example, in the lipid composition of the mitochondria, the plasma membrane, and so forth. I just think it is worth mentioning because of the possibility—well, the probability—that much of our attention about the mechanism of hormone action is going to be directed in the near future toward the function of various membranes—cellular and intracellular.

ARNAUD: I think this is going to be a very profitable approach. I hope that you will couple some studies that you are going to be doing on lipids with ion flux studies, because I think this is most important. The correlation is going to be terribly important, especially the time correlation.



PECK: The point that you make is a good one. Interpretation of these kinds of data must be done with extreme caution. In this kind of system, there is no telling what comes first, the chicken or the egg.

NICHOLS: I think you are involved, too, in the question of whether you may be dealing simply with transfer of precursors.

HOLTZER: In this context, I should like to mention some experiments that Dr. Nameroff and I are engaged in. It is a demonstration of what you can do with tissue culture, worrying about cell membranes.

Yesterday I talked of how liberated cartilage cells, if grown in a dense culture, would form a layer of cartilage within 5 or 6 days (ref. 82). Now, if freshly liberated cartilage cells are added to these cultures, the added cells are not induced to reenter the mitotic cycle as they would be if they were plated out on glass. Chondrocytes plated on glass rapidly flatten and their cell surface increases sixfold in a matter of hours. These are the cells that are induced to make DNA. However, when these cells are added to a sheet of preformed cartilage, they do not flatten and they are not induced to make DNA. They remain round and make chondroitin sulfate. Our current interpretation is that the permeability of the rounded chondrocyte is quite different from that of a stretched-out one, and that this type of difference leads to profound differences in the activities of the respective cells.

FREMONT-SMITH: You are showing how important the environment is in the specialized functions of the cell. I had to throw my concept in on that.

NICHOLS: You wanted to add a shape to that.

FREMONT-SMITH: The environment determines the shape in this case.

NICHOLS: May I talk about what may be an example of a neutral induction system?

URIST: Please do.

NICHOLS: Yesterday I started to tell you something about multiple myeloma, which I would like to suggest may be an example of an induction system at work.

Multiple myeloma, for those of you who are not clinicians, is a disease which affects bones. Bone destruction is seen which is thought to be the result of an invasion of an abnormal form of a connective tissue cell—the plasma cell. There are various bone lesions ranging from “osteoporosis,” which is just osteopenia by X-ray, to multiple punched-out lesions. The latter contain masses of abnormal plasma cells and are called plasmacytomas. The bone cells themselves appear histologically normal.

The data I would like to show are of the same sort as those I showed before and are taken from a recent publication (ref. 228). Included

are data concerning bone, the myelomatous marrow that has been washed out of the bone, and plasmacytomas as examples of pure cultures of abnormal plasma cells. The reason for studying marrow and plasmacytomas as well as bone cells was that the changes we hoped to find in the bone might be simply due to contaminating myeloma cells—a fact which might go unrecognized if we did not learn about the plasma cells.

Figure 154 summarizes the data on bone-cell metabolism. The shaded areas indicate the range covered by two standard deviations above and below the mean normal value as before. Individual patients are shown by number so that the combination of metabolic values for any individual may be readily identified.

Clearly, there are patients in this group whose bone cell  $O_2$  uptake and lactate production are abnormally active, but the data are too scattered to allow a characteristics pattern to be identified. The data concerning proline metabolism are those I would like to emphasize. Seven of the 10 showed slight-to-marked increase in retention of proline label in the bone cells, but only two showed any evidence of the concomitant increase in labeling of the collagen of the samples, and of these only one was clearly high. While increased labeling from glucose was also present, the dichotomy between the changes in cellular and collagen labeling was not preeminent.

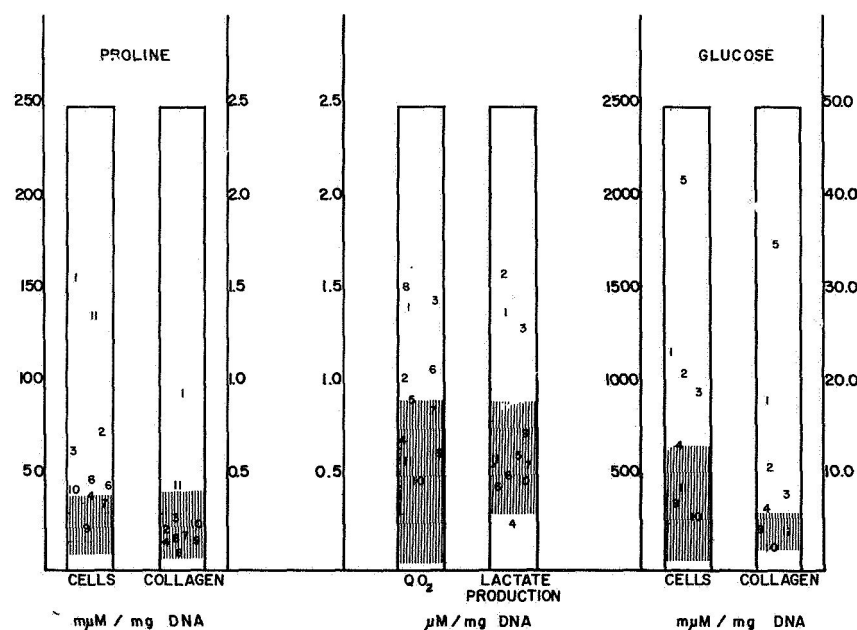


FIGURE 154. Bone metabolism in multiple myeloma.

These findings raised two questions: What was the excess proline label in? What was the contribution of marrow contaminants to these changes? The answer is largely in a cell fraction insoluble in 3 percent trichloroacetic acid, suggesting incorporation into a macromolecule—perhaps protein—but this material remains to be identified.

The answer to the second question is in part supplied by the data in figure 155. Most of these data fall within the range of values obtained in the few normal samples of marrow which we have examined. Of particular importance was the fact that the proline incorporation into myelomatous marrow cells was not different from controls. Since clear correlations between changes in marrow and bone cells could not be found, it has been tentatively concluded that marrow probably contributed little to the apparent changes which we found in bone-cell metabolism.

One other observation from this study might be of interest, although it is negative. The possibility that the excess proline retained in the cells might be in a protein suggested to us the possibility that collagenase is being made in excess in these cells and might be responsible for the excess bone breakdown. Collagenase activity was, therefore, measured. The data obtained are shown in figure 156. Again, the normal range is shown by the shaded area. Collagenase activity was normal

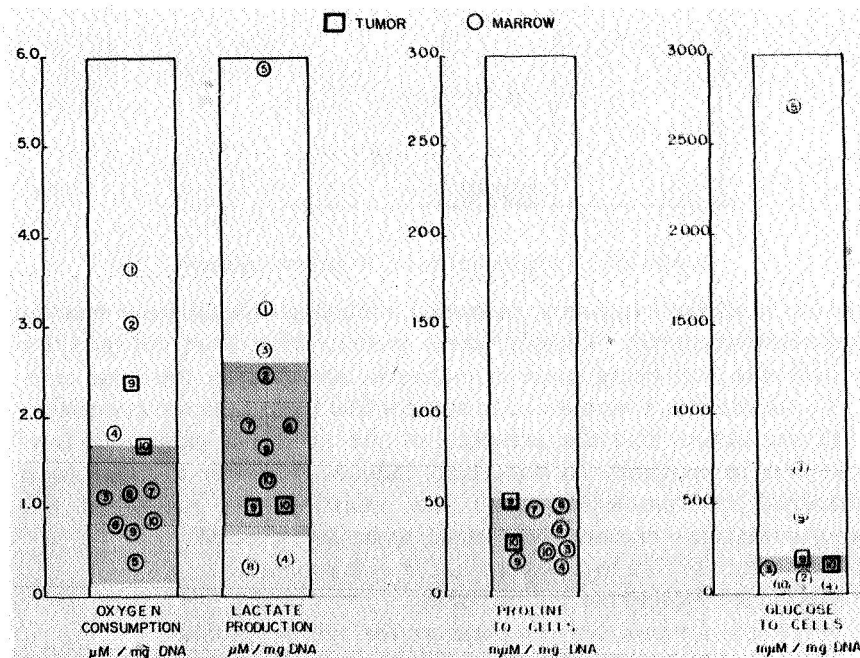


FIGURE 155. Marrow metabolism in multiple myeloma.

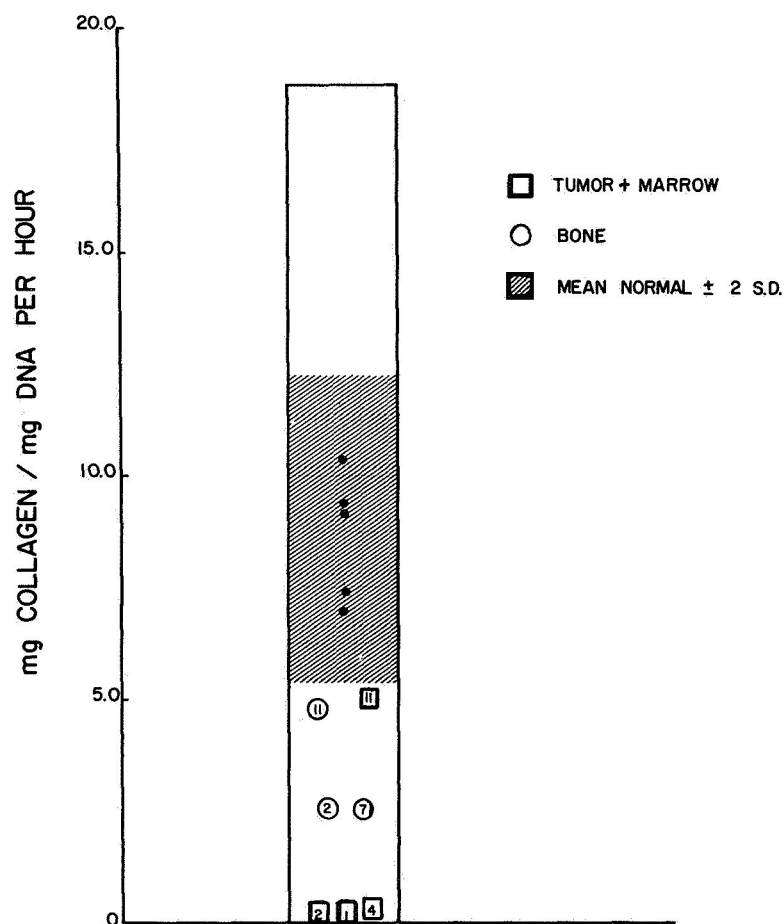


FIGURE 156. Collagenase activity in multiple myeloma.

or low in all the specimens examined, including myelomatous marrow and plasmacytomas. Clearly, some protein other than collagen and collagenase was being made in excessive amounts by the bone cells.

To summarize, I would like to suggest that these data are compatible with the notion that the presence of the abnormal plasma cells of myeloma in proximity to bone cells induces the latter to make some abnormal macromolecule. The real question—the nature of this macromolecule—remains, of course, to be investigated.

HOLTZER: Is that hydroxyproline or is it proline?

NICHOLS: Proline carried the label.

HOLTZER: I mean, did you measure for hydroxyproline?

NICHOLS: Not as such. The data are simply on incorporation of proline label without much more discrimination.

HOLTZER: Right.

NICHOLS: We have shown that the label in the collagen when derived from the proline is present in the proline and hydroxyproline. Is that what you mean?

HOLTZER: In that material, or in that protein, was there proline or was there hydroxyproline—

NICHOLS: In the cells? I do not know. I just have not looked.

SAXÉN: Can you exclude the possibility that your marrow samples were contaminated by the plasma cells?

NICHOLS: They contained plasma cells. The relative properties varied somewhat, but the data that are obtained from pure plasmacytoma are very similar, so that it probably does not matter.

HOLTZER: My question is, Is that making hydroxyproline-containing protein?

NICHOLS: I cannot answer that—I do not know.

HOLTZER: Then why do you say that the bone cells are making something? Why is it not just the myeloma cells that are making something?

NICHOLS: Because when you look at myeloma cells or myelomatous marrow, you find that the incorporation of proline into these cells is actually lower than the incorporation of proline into cells derived from the bone from which the marrow has been washed. So it seems unlikely that marrow-cell contaminants are responsible. It is not solid proof, of course.

BÉLANGER: Dr. Nichols, whenever we have a tumor anywhere, particularly a tumor of long duration and a tumor which would produce antigenic reactions in the organism, is it not possible that the system will react locally by producing cells that tend to destroy this tumor, and among those we would have, for instance, histiocytes? And if you have a large number of histiocytes in bone or in tumors or elsewhere, these are very well known to be rich in lysosomes, so they would increase, probably, the quantity of proteolytic enzymes which would be present in such tissue? This would be an important factor wherever you have tumor.

NICHOLS: This is perfectly true, and we have not measured other proteolytic enzymes besides collagenase so far. We think it would be amusing to see whether we could identify by electrophoresis or immunologic techniques a protein containing this excess label. Myeloma is of great advantage in this sort of work, since characteristic abnormal proteins are to be found in the plasma. Perhaps this is being made by bone cells operating under orders from the plasma cells.

PECK: The striking thing about myelomatous bone destruction is that it is unaccompanied by a response on the part of the bone that can be demonstrated histologically. Osteoblastic activity would seem

to be inhibited, and there is certainly no significant histiocytic response to support myeloma that can be demonstrated in these cavities.

I have a question just for the record. How many of these patients were receiving alkylating agents at the time of biopsy?

NICHOLS: The data on marrow (fig. 155) were from 11 patients, and of that group, if my memory serves me, there were two who were receiving alkylating agents, one or the other of the sarcolysins, at the time of biopsy. Some others had received sarcolysin. None of these patients was receiving fluoride at the time of biopsy. Since then, five have been on fluoride, of which we have biopsied four.

There are several intriguing things about the response of myeloma patients to fluoride. I do not have the faintest notion what they mean. I am sorry to say they may lend fuel to the fire of the idea that you must have proliferating tissue to get induction. Patients with multiple myeloma to whom one gives a therapeutic dose of fluoride develop fluorosis in a period of time which is much shorter than the time in normal subjects. It takes the normal subject, or the osteoporotic patient, at least 6 months to show a change in alkaline phosphatase and 9 to 12 months to show a roentgenographic change, whereas the myelomatous patients will show these changes in a matter of 6 to 8 weeks, including roentgenographic changes. We had one man who developed very marked widening of his trabecula in 2½ months.

PECK: To the best of my knowledge, the only cases that Dr. Cohen has published with respect to this response to fluoride were also receiving massive doses of androgens (ref. 229). Does this 6-week response occur in the absence of concomitant therapy?

NICHOLS: In the absence of androgen, yes. In the absence of calcium, we do not really know. All of these patients have been given calcium and some vitamin D, too. I do not know exactly how much of this is related to that response.

ARNAUD: Did any of them become hypercalcemic during the period of study?

NICHOLS: No. All of these patients were normocalcemic. It is interesting that all of the patients on fluoride have shown an inhibition of uptake of proline into the cells and a stimulation of proline into collagen, which is sort of a reversal of the normal pattern in our osteoporotic patients. In other words, the fluoride seems to return their bone metabolic pattern toward normal except for one man who, on the third biopsy taken in the final stages of his illness, showed a return to an abnormally active pattern after having previously shown the usual inhibition.

BAUER: I have recently reviewed some evidence, based on tracer studies with <sup>47</sup>Ca and <sup>85</sup>Sr, that myeloma does not elicit the intensive

repair reaction usually encountered in bone tissue hit by tumor, infection, or fracture (ref. 230).

NICHOLS: This is an important point, with relation to the biology of skeletal tissue at large, because one wonders whether we are seeing normal bone cells that have been induced to make some abnormal product. If this could be shown, then by what means? We may have a good example there of an induction system due to tumor. One also is tempted to ask, "Is the inducer that made the plasma cell abnormal the same one that made the bone cell abnormal?"

URIST: Would you like me to show you how to turn induction systems on or off?

MCLEAN: Yes.

URIST: This can be demonstrated by experiments with an implant of decalcified bone matrix (refs. 151 and 231). We have seen slides from some of our experiments on decalcified bone-matrix implants. Now I would like to summarize our findings of the chemical aspects of this process.

In collaboration with Barry Silverman, Juan de la Sierra, and other orthopedic research fellows at UCLA, we are testing decalcified bone treated or denatured in various ways by many different chemical agents. At this time, it can be reported that bone induction is inhibited if 0.6 *N* HCl-decalcified matrix is further denatured by: (1) boiling at 100° C, but not by heating, for 5 minutes at temperatures between 50° and 90° C; (2) incubation in solutions by pronase, ficin, papain, chymopapain, elastase, collagenase, but not by hyaluronidase, acid phosphatase, or alkaline phosphatase; treatment with  $\beta$ -propiolactone for sterilization, fluorodinitrophenol for blocking  $\epsilon$ -amino and other reactive groups, or HNO<sub>2</sub> or HNO<sub>3</sub> for deamination of the tissue proteins, or metal ions with special toxic effects, e.g., BeCl<sub>2</sub>.

Bone induction is either enhanced, or not perceptibly inhibited, by: (1) freezing at -30° C; (2) lyophilization at -70° C; (3) sterilization in 70 percent alcohol; (4) defatting with alcohol, acetone, ether, or detergents such as pHisoHex; (5) extraction in concentrated salt solutions such as 0.5 *M* NaCl, or CaCl<sub>2</sub>, to remove mucoproteins and lipoproteins; (6) treatment with solutions of various carboxylic acid blocking reagents such as toluidine blue, protamine sulfate, PbCl<sub>2</sub>, FeCl<sub>3</sub>, CaCl<sub>2</sub>, and others.

These experiments demonstrate something about the influence of the chemical composition of the implant upon the cells that grow in and repopulate the interstices of the implant. They do not demonstrate whether chemical substances are transferred from the denatured matrix to the cytoplasm of the various cells that grow into the area of the implant. We performed an experiment with implants of 0.6 *N* HCl-

decalcified bone in a Millipore chamber, but observed no bone formation either inside or outside the Millipore membrane. Bone formed inside in one instance when the cell grew through a crack in the filter and gained contact with the matrix in one place. (See fig. 86(b).) Therefore, we may assume the inducer is mobilized by contact with cells.

SAXÉN: What kind of Millipore filter was it? Do you remember its pore size?

URIST: Pore size was 0.45 micron, 150 microns in thickness, the same as employed by Goldhaber (ref. 232).

To follow the pathways of proliferation of cells,  $^3\text{H}$ -thymidine was injected into the host, and grain counts were made of labeled cells in various parts of the implant. The percentage of labeled cells was low in the area of histiocytes, the interstices of the old matrix, and very high in the progenitor cells around sprouting blood vessels, specifically in areas of active osteogenesis. In this respect, the picture resembled the distribution of labeled cells in the metaphysis of a growing bone.

To assemble our observations and describe the process in terms of modern concepts of embryonic induction, table XXXV illustrates a

TABLE XXXV  
BONE AUTOINDUCTION: CELL-INDUCTION SEQUENCES

Days after implantation	Before induction		Inducing matrix and microenvironmental variables	After induction	
	Inducing cell	Responding cell		Responding cell	Differentiated cell
1 to 22.....	A	B	$\left( \begin{array}{l} \text{CO}_2 \text{ tension,} \\ \text{O}_2 \text{ saturation,} \\ \text{Cell metabolites} \end{array} \right)$	B	D
	Wandering histiocyte	Fixed histiocyte	$\left( \begin{array}{l} \text{Open excavation chamber,} \\ \text{High O}_2 \text{ saturation,} \\ \text{Low CO}_2 \text{ tension,} \\ \text{Other cell metabolic products} \end{array} \right)$	Fixed histiocyte	Osteoprogenitor cell and osteoblast
22 to 30.....	A	B	$\left( \begin{array}{l} \text{CO}_2 \text{ and O}_2 \text{ saturation,} \\ \text{Cell metabolites} \end{array} \right)$	B	C
	Wandering histiocyte	Fixed histiocyte	$\left( \begin{array}{l} \text{Compaction of cells in closed} \\ \text{vascular channel,} \\ \text{High CO}_2 \text{ tension,} \\ \text{Low O}_2 \text{ saturation,} \\ \text{Other cell metabolic products} \end{array} \right)$	Fixed histiocyte	Chondroprogenitor cell and chondroblast
22 to 30.....	C	B	(Vascularized excavation chamber)	B	D
	Chondrocyte + connective tissue cell	Perivascular connective tissue cell	$\left( \begin{array}{l} \text{Absorption cavities,} \\ \text{High CO}_2 \text{ tension,} \\ \text{Low O}_2 \text{ saturation} \end{array} \right)$	Perivascular connective tissue cell	Osteoprogenitor cell and osteoblast

Adapted from ref. 151.



theory of autoinduction in which: (1) the decalcifying matrix is an inducing surface; (2) the progeny of the first generation of cells to populate the implant are the inducing cells; (3) young perivascular connective tissue cells are the responding cells; (4) progenitor cells are the induced cells; and (5) cell specialization for cartilage formation and for bone formation is the culmination of cell migration, a series of mitotic divisions, and the product of the interaction of the cell and its microenvironment. Osteogenesis is, therefore, not a triggered event, but depends upon a sequence of cell changes which occurs in a bone-matrix implant over a period of 3 weeks. The surface of the inducing matrix could affect the cell membrane, transfer material to the ribosomal system or the genetic structure, but we have no information at this time either to implicate or reject a reaction at any one of the three sites in the cell. Osteogenesis developed in this way should be regarded as autoinduction insofar as both inducing and responding cells originate in the same individual.

Neither the nature of the inducer nor the local chemical mechanisms of induction is known, but present investigations promise to produce an important advance in knowledge of bone physiology. The experiments mentioned deal chiefly with inducing surfaces of the matrix. Our work at present also deals with systemic factors such as the competence of the responding cells. We are now finding, for example, that an aged host produces relatively little bone induction and always after some delay. Antimetabolites, antibiotics, and other cell toxins are being tested to determine the action of various ultramicroscopic organelles in bone induction.

PRITCHARD: That was very nicely told, Dr. Urist.

URIST: Thank you. If there are no further comments, we will now adjourn this meeting.

ROBINSON: Before we go, I would like to thank Dr. Fremont-Smith and the New York Academy of Sciences for a very pleasant meeting here, and particularly Dr. Urist and the others who have given us useful information.



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